Nutritional plasticity and evolutionary divergence in the *Drosophila* ovary

Cláudia Carolina de Almeida Mendes

Dissertation presented to obtain the Ph.D degree in Developmental Biology

Instituto de Tecnologia Química e Biológica António Xavier | Universidade Nova de Lisboa

Oeiras,
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This thesis is dedicated to

My parents, António Mendes and Luciana Cruz,

for always encouraging my creativity and curiosity.

Declaração/Declaration

Declaro que esta dissertação é o resultado do meu próprio trabalho desenvolvido entre Abril de 2011 e Janeiro de 2015 no laboratório da Dra. Christen Mirth, Instituto Gulbenkian de Ciência em Oeiras, Portugal, com co-orientação do Dr. Élio Sucena do Instituto Gulbenkian de Ciência, Oeiras, Portugal. Este doutoramento foi realizado no âmbito do Programa Gulbenkian de Doutoramento (edição 2010-2011). Parte do capítulo 1 foi publicado no Frontiers in Physiology como "Mechanisms regulating nutrition-dependent developmental plasticity through organ-specific effects in insects", T. Koyama, C.C. Mendes and C.K. Mirth (2013). O capítulo 2 e 3 estão integrados num manuscrito submetido para publicação com autoria de C.C. Mendes e C.K. Mirth. O capítulo 4 está integrado num manuscrito em preparação com autoria C.C. Mendes, E. Sucena e C.K. Mirth.

I declare that this dissertation is a result of my own research carried out between April 2011 and January 2015 in the laboratory of Dr. Christen Mirth, Instituto Gulbenkian de Ciência in Oeiras, Portugal, with the co-supervision of Dr. Élio Sucena of the Instituto Gulbenkian de Ciência, Oeiras, Portugal. Part of chapter 1 has been published in Frontiers in Physiology entitled "Mechanisms regulating nutrition-dependent developmental plasticity through organ-specific effects in insects", T. Koyama, C.C. Mendes and C.K. Mirth (2013). Chapter 2 and 3 are part of a manuscript submitted for publication, authored by C.C. Mendes and C.K. Mirth. Chapter 4 is part of a manuscript in preparation, authored by C.C. Mendes, E. Sucena and C.K. Mirth.

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SUMMARY

The environment can modify developmental trajectories and generate a range of distinct phenotypes without altering an organism's genome, a widespread phenomenon called developmental plasticity. The past decades have seen a resurgent interest in understanding how developmental plasticity contributes to evolutionary processes, as it can produce phenotypic variation among individuals and facilitate diversification among populations that inhabit distinct ecological niches. To better understand the importance of plastic responses for evolutionary change, we need to explore how the environment alters development to produce phenotypic variation and then compare this to how genetic variation influences these same developmental processes.

My thesis work explored the developmental mechanisms underlying both plasticity and subspecies-specific variation in ovariole number, a major determinant of female reproductive capacity, in *Drosophila*. Ovariole number is determined during third instar (L3) larval stages and begins with the differentiation of terminal filament cells (TFCs) that gradually intercalate into stacks called terminal filaments (TFs). The number of TFs at pupariation directly determines the number of ovarioles. The developmental processes underlying TF formation are know to vary both with environmental conditions, like nutrition, and between species.

I first addressed how nutrition influences ovariole number in *D. melanogaster*. By manipulating nutrition at specific stages during L3 larval development, I found that ovariole number exhibits two phases of sensitivity to nutrition (Chapter 2). These two phases are separated by

the nutrition-dependent developmental transition known as critical weight. When larvae are poorly fed during the first phase of sensitivity, ovary growth arrests and the onset of TFC differentiation is strongly delayed, resulting in a severe reduction in ovariole number. On the other hand, the effects on ovariole number in larvae that are malnourished during the second phase are more modest; ovary growth and the formation of new TFs continue, although at a reduced rate relative to well-fed larvae.

Secondly, I determined the role of two hormonal pathways, the insulin/insulin-like growth factor signalling (IIS) and ecdysone signalling pathways, in regulating the nutritional sensitivity of the ovary (Chapter 3). My results indicate that both pathways regulate the nutritional-sensitive onset of TFC differentiation, with ecdysone signalling playing a pivotal role in this process. Conversely, IIS, and to a lesser extent, ecdysone signalling coordinate the rate of TF formation and of ovary growth with nutritional conditions.

Lastly, I investigated the developmental changes that give rise to differences in ovariole number between two subspecies of *D. mojavensis*, the *D. moj. sonorensis* and *D. moj. wrigleyi* (Chapter 4). As these subspecies inhabit geographically isolated areas and breed in distinct host cacti, they provide a unique opportunity to investigate the early events associated with morphological diversification. Based on my detailed characterizations of ovary development, I found that differences in the rate of ovary growth can explain much of the variation in ovariole number between *D. moj. sonorensis* and *D. moj. wrigleyi.* From these findings, I propose that evolutionary changes in the activity of IIS could underlie the differences in ovary growth, and consequently ovariole number, between these subspecies (Chapter 5).

In summary, my results underscore the importance of hormonal pathways in coordinating stage-specific developmental processes with environmental conditions, and specifically suggest that changes in the activity of hormonal pathways can account for plastic responses, and potentially also for evolutionary diversification.

The powerful developmental approach embraced in this thesis may be useful to investigate how other morphological traits respond to environmental variation, and could provide significant insights to unravel one of the most captivating mysteries of biology; that of the origin of diversity in living things.

SUMÁRIO

O ambiente pode alterar o desenvolvimento de um organismo e criar uma variedade de fenótipos sem alterar o seu genoma. Este fenómeno, extremamente comum na natureza, é denominado plasticidade. Nas últimas décadas, o interesse em compreender como a plasticidade pode contribuir para os processos evolutivos tem vindo a crescer. É um interesse preenchido ao facto de a plasticidade ser capaz de produzir variação fenotípica entre indivíduos e facilitar a diversificação entre populações que habitam diferentes nichos ecológicos. Para melhor compreender a importância da plasticidade na evolução, é necessário explorar de que forma o ambiente altera o desenvolvimento para produzir diversos fenótipos, e identificar se alterações semelhantes no desenvolvimento são responsáveis pela variação fenotípica entre espécies.

Esta tese teve como objetivo interpretar os mecanismos de desenvolvimento, que estão na base de, quer de respostas plásticas no número de ovaríolos de *Drosophila*, quer de diferenças neste número resultante de variação genética entre subespécies de *Drosophila*. O número de ovaríolos influencia a capacidade reprodutiva da fêmea e é determinado durante o terceiro estágio larvar. O processo de formação dos ovaríolos começa com a diferenciação de 'células dos filamentos terminais' (TFCs), que gradualmente se intercalam, formando pilhas de células denominadas 'filamentos terminais' (TFs). O número de TFs no momento da pupariação iguala o número de ovaríolos no adulto. Este processo pode variar com as condições ambientais da larva, como, por exemplo, com a nutrição, e com a variação genética entre espécies.

Primeiramente investiguei como a nutrição influencia o número de ovaríolos em *D. melanogaster*. Ao manipular a nutrição em diferentes alturas do terceiro estágio larval, demonstrei que o número de ovaríolos exibe dois períodos sensíveis à nutrição (Capítulo 2). Estes são separados pela transição de desenvolvimento conhecida como peso crítico. Quando as larvas são submetidas a um défice alimentar durante o primeiro período sensível, o crescimento do ovário é reprimido e o início da diferenciação dos TFCs é extremamente atrasado, resultando daí, uma severa redução no número de ovaríolos. Por outro lado, os efeitos no número de ovaríolos em, larvas que são mal nutridas, durante o segundo período sensível à nutrição são mais moderados; o crescimento do ovário e a formação de novos TFs continua. No entanto, a sua taxa de progressão é reduzida relativamente a larvas bem nutridas. De seguida, explorei a função de duas vias de sinalização hormonal, a via da insulina e a via da ecdisona, na regulação da resposta nutricional do ovário (Capítulo 3). Estes resultados indicam que ambas as vias regulam o início da diferenciação dos TFCs, tendo a via da ecdisona um papel fulcral neste processo. Contrariamente, ambas as vias, a da insulina, e, em menor grau a da ecdisona, regulam as taxas de formação de TFs e do crescimento do ovário em resposta às condições nutricionais.

Finalmente, explorei possíveis alterações no desenvolvimento que pudessem explicar as diferenças observadas no número de ovaríolos entre duas subespecies de *D. mojavensis*, a *D. moj. sonorensis* e a *D. moj. wrigleyi* (Capítulo 4). Estas subespecies habitam áreas geograficamente isoladas e desenvolvem-se em cactus distintos, providenciando uma oportunidade única para investigar os primeiros eventos associados com a diversificação morfológica. Baseado nas caracterizações detalhadas do desenvolvimento ovárico que efetuei, demonstrei que as diferenças na taxa de crescimento do ovário podem explicar, em grande parte, as diferenças no número de ovaríolos entre a *D. moj. sonorensis* e a *D. moj. wrigleyi*. Tendo em conta esta observação, propus que mudanças evolutivas na atividade da via da insulina poderão estar na base das diferenças da taxa de crescimento do ovário, e, consequentemente, no número de ovaríolos entre as duas subespecies (Capítulo 5).

Resumindo, estes resultados revelam a importância das vias de sinalização hormonal na regulação de processos que ocorrem em períodos específicos do desenvolvimento, e na sua coordenação com as condições ambientais. Além disso, sugerem também que, mudanças na atividade de vias de sinalização hormonal são responsáveis, tanto pela resposta plástica como, potencialmente também pela diversificação evolutiva.

A poderosa abordagem focada no desenvolvimento, que foi utilizada nesta minha tese poderá ser útil para investigar como outras características morfológicas respondem à variação ambiental. Desta forma, poderá auxiliar a resolver um dos mistérios mais cativantes da biologia: o da origem da diversidade de formas de vida.

1

GENERAL INTRODUCTION

"Fasten your seatbelts. It's going to be a bumpy night."

– from the film *All about Eve* (1950)

1.1 The mystery of biodiversity

When we look carefully at the natural world, we cannot help but notice the wonder of living things. From the astonishing beauty and diversity of species that inhabit even some of the most inhospitable places on Earth to the spectacular and intricate machinery of the cells that can only be appreciated at the molecular level. But, how did such diversity and complexity come to be? The theory of evolution developed by Charles Darwin and Alfred Russel Wallace drastically changed our perception of how life forms diversify. After the widely scientific acceptance of the theory of evolution during the 1930s and 1940s – when empirical and theoretical work recognized genes as the unit of evolutionary change by means of natural selection –, “most evolutionary geneticists would agree that the major problems of the field have been solved” (Charlesworth, 1996).

Yet, an emerging paradigm of how living things diversify has recently put forward by researchers from different disciplines, including genetics, developmental biology, physiology and ecology. This comprehensive view argues that changes that occur during an organism’s development as a result of the delicate interplay between genes and the external environment should be recognized as causes of evolutionary change (West-Eberhard, 2003; Stearns, 1989; Moczek, 2012; Laland et al., 2014). From this broader vision of evolution, a particularly exciting concept has resurged that enhances our understanding of the origins of phenotypic variation; that of developmental plasticity. This widespread phenomena refers to the ability of an organism to change its developmental trajectories in response to environmental variation and generate a range of phenotypes without altering its genome (West-Eberhard, 2003; Stearns, 1989). Such environmentally-induced changes were once seen as nuisance and oddities that complicated evolutionary and developmental studies, but within the last decade, a renewed interest in how developmental plasticity might contribute to evolutionary diversification has grown tremendously (West-Eberhard, 2003; Stearns, 1989; Wund, 2012; Laland et al., 2014; Beldade et al., 2011).

The aim of this thesis was to enrich our knowledge of the importance of developmental plasticity in generating morphological diversity within and

between species. In this introductory chapter, I therefore introduce the concept of developmental plasticity and discuss what is known about its contribution to evolutionary processes. I then focus on the importance of environmental sensitivity during development (with emphasis on insects), and how changes in the timing and amount of hormone production account for many, if not all, environmentally-induced phenotypes. Finally, I conclude the chapter with a discussion regarding some unsolved issues in our understanding of developmental plasticity and how this thesis will address those gaps.

1.2 Developmental plasticity and evolutionary diversification

A plethora of environmental cues can mould the developmental programs of an organism and lead to the production of distinct phenotypes (Beldade et al., 2011). The resulting phenotypes can range from gradual changes, such as temperature-induced differences in body and wing size in the fruit fly (Partridge et al., 1994), to dramatically distinct polyphenic morphs, such as the gregarious and solitarious forms of several locusts species (Rogers et al., 2014). Such morphological responses are induced during specific developmental stages and are typically irreversible, while behavioural and physiological traits tend to be flexible and can be rapidly reversed (reviewed in (Whitman and Agrawal, 2009)). Importantly, not all environmentally-induced phenotypes are adaptive, and some can even be maladaptive (Price et al., 2003; Whitman and Agrawal, 2009). Nevertheless, because it provides a range of phenotypic responses to changes in the environment, plasticity can facilitate phenotypic divergence among individuals and ultimately guide evolutionary change (reviewed in (Pfennig et al., 2010)).

The implications of developmental plasticity on evolution have been illustrated in myriad review articles, books and research papers (Beldade et al., 2011; Pfennig et al., 2010; Pigliucci, 2001; Pigliucci et al., 2006; West-Eberhard, 2003; Whitman and Agrawal, 2009). Here, instead of providing an extensive review of the literature, I will briefly

outline a potential scenario by which plasticity promotes phenotypic diversification. As selection acts on phenotypes rather than genotypes, buffering mechanisms during development, usually referred as canalization or robustness, may prevent selection from acting on newly arising mutations of small effect and facilitate their accumulation within a range of environmental conditions (Flatt, 2005). Such genetic variation is often described as cryptic genetic variation, in which genetic variants have little or no effect on phenotypic outcome (Gibson and Dworkin, 2004; Paaby and Rockman, 2014; Schlichting, 2008). In response to novel environmental stimuli (e.g. after migration to a new ecological niche), buffering mechanisms could be disrupted allowing previously cryptic genetic variation to become expressed as a broad range of novel phenotypic variants: some will be maladaptive, while other may allow a population to persist in the new environment. Such unmasking of heritable variation enables natural selection to operate; that is, gene combinations and regulatory networks that stabilize and integrate the induced, favoured phenotype are gradually spread and fixed across the population through a process called genetic accommodation [1] (Moczek, 2007; Pfennig et al., 2010; West-Eberhard, 2003).

Under some circumstances, the environmental stimuli may no longer be required for the expression of the induced, favoured phenotype, resulting in the loss of environmental sensitivity by means of genetic assimilation (Pigliucci et al., 2006; Waddington, 1959). This further suggests that developmental plasticity itself can evolve. In fact, the ability of living things to alter their development in response to environmental cues is probably the ancestral state, with selection then acting to maintain or buffer environmental effects (Nijhout, 2003b).

Taken together, the environment can unravel hidden and novel phenotypes that become expressed constitutively in a population, thus promoting diversity among populations inhabiting alternative niches and ultimately guiding evolutionary change. Importantly, the phenotypic divergence between individuals "begins not with genetic change, but

[1] Novel phenotypes generated by mutation also become stabilized through the process of genetic accommodation (West-Eberhard, 2003).

with environmentally induced change to the phenotype" ((Whitman and Agrawal, 2009), pp.39). Current empirical and theoretical studies support a role of developmental plasticity in evolution (Price et al., 2003; Standen et al., 2014; Susoy et al., 2015), however, there are still many open questions, particularly, on the nature and release of cryptic genetic variation (Gibson and Dworkin, 2004; Paaby and Rockman, 2014; Schlichting, 2008) and on the molecular and developmental mechanisms that allow an induced phenotype to become constitutively expressed (Moczek, 2007; Suzuki and Nijhout, 2006; Nijhout, 2008; West-Eberhard, 2003).

The study of developmental plasticity is not just a scientific dispute on how life forms evolved (Laland et al., 2014). A better comprehension of how the environment moulds phenotypes will have important implications in our understanding on how organisms can cope with global climate change (Beldade et al., 2011; Chevin et al., 2010); how invasive species can rapidly colonize new environments and threaten the diversity of local species (Davidson et al., 2011; Moczek, 2007); and how complex human diseases, such as cancer and diabetes, arise (Feinberg, 2007). Exciting and insightful understanding can therefore be drawn from the study of developmental plasticity and shed a new light on current issues in modern science.

1.3 Developmental sensitivity to environmental cues

Developmental processes are modified by numerous external environmental factors, including abiotic (e.g. nutrition and temperature) and biotic (e.g. population density and parasites) signals. Such environmental signals typically trigger a simultaneous change in the development of several different traits. However, the degree of plasticity is often specific to individual traits and environmental conditions (Mirth and Shingleton, 2012). The variation in an individual trait in response to environmental conditions is usually described as a reaction norm – graphical representations where phenotypic variation is plotted over an

array of environments (Figure 1.1) (Schlichting and Pigliucci, 1998). In the fruit fly *Drosophila melanogaster*, for instance, different organs respond differently to the same environmental signal (Shingleton et al., 2009). Larvae reared in poor nutritional conditions show dramatic reductions in the size of their wings, palps and legs (Shingleton et al., 2005, 2009), whereas other organs, such as the male genitalia and the central nervous system (CNS), vary little with nutritional changes (Shingleton et al., 2005; Tang et al., 2011).

On the other hand, a given trait may show different plastic responses depending on the environmental cue (Shingleton et al., 2009). The size of the *Drosophila* ovary is determined by the number of ovarioles, which are the functional and discrete units where oogenesis takes place (King et al., 1968). While nutrition and ovariole number exhibit a linear and positive reaction norm (i.e. rich nutritional environments enable the formation of an optimal number of ovarioles) (Bergland et al., 2008), rearing larvae either at higher or at lower temperatures than the optimal (25ºC) reduces ovariole number, resulting in a relationship with a bell shaped form (Klepsatel et al., 2013a).

The degree of plasticity of an individual trait also greatly depends on developmental windows of environmental sensitivity during which environmental cues can alter the course of developmental trajectories (Pigliucci, 2001). When such developmental windows, also known as critical periods, are surpassed, changes in environmental conditions induce more modest alterations in the related trait. Critical periods may exist simply because developmental processes are often continuous and irreversible (Nijhout, 1999). This is particularly relevant in holometabolous insects, such as lepidopterans (e.g. butterflies and moths) and dipterans (e.g. fruit flies), where adult body size and many other adult traits are determined during larval stages.

One of the best examples of the importance of critical periods in determining phenotypic outcomes is the butterfly *Bicyclus anynana*. This tropical butterfly develops two seasonal morphs in response to temperature to avoid predation: a wet-season phenotype with conspicuous marginal eyespots in the ventral hindwings and a dry-season form that have very

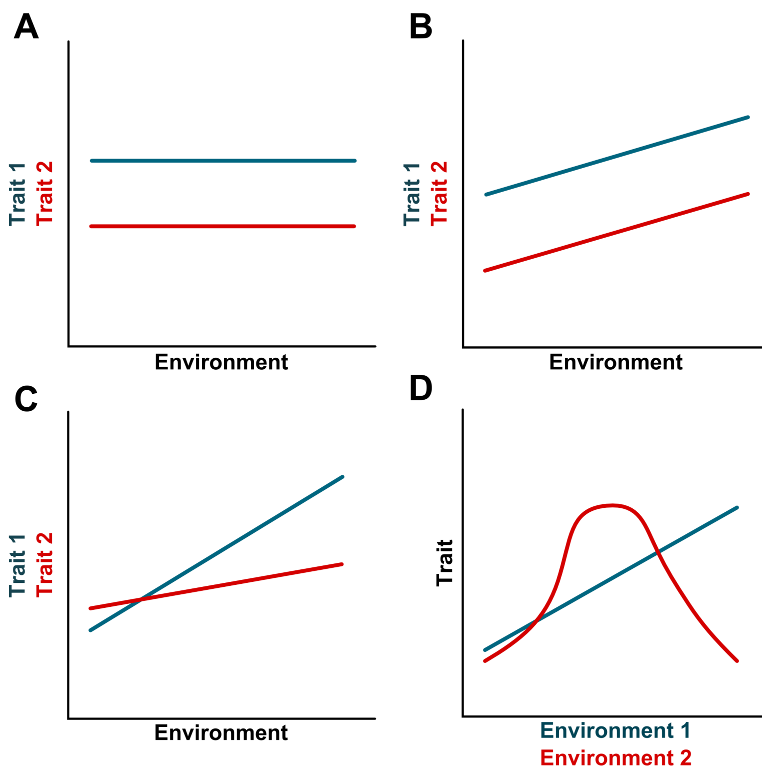

Figure 1.1: The relantionship between phenotype and the environment. (A) Average size values of two traits (blue and red lines) are different due to genetic variation. Plasticity is absent. (B) Both traits respond to environmental variation and the degree of their plastic response is similar. Plasticity is present, but genetic variation in plasticity is absent. For example, wing and body size in *D. melanogaster*. (C) The slopes of the reaction norms are different, suggesting that the two traits respond differently to the same environmental cue. Plasticity and genetic variation in plasticity are present. For example, male genitalia and wing size in *D.melanogaster*. (D) A given trait may show distinct plastic responses depending on the environmental cue. For example, ovariole number in *D.melanogaster*. See text for more information.

small eyespots. Temperature shift experiments at specific developmental points revealed that the critical period in which temperature can induce changes in wing pattern occurs late in larval development (Kooi and Brakefield, 1999). Further examples have been described in other polyphenic butterflies (Nijhout, 2003b). These studies are beginning to allow us to piece together how critical periods of environmental sensitivity are regulated.

1.4 Hormonal mechanisms of developmental plasticity

Recent research has revealed that environmental factors often modulate developmental trajectories to produce distinct phenotypes by controlling which, when, and how strongly genes are expressed during development. The action of hormones is perhaps one of the best-understood mechanisms mediating developmental plasticity. Hormones are known to integrate information from the external environment and regulate multiple developmental processes throughout the entire organism: some bind directly to transcription factors and activate the expression of specific genes (Baniahmad and Tsai, 1993), others activate a series of intercellular signalling cascades that regulate growth (Wu and Brown, 2006) and even others change the DNA methylation profiles in the genome, regulating which genes are expressed (Beldade et al., 2011; Snell-Rood et al., 2013).

An integrated picture of how hormones link environmental variation with developmental changes has largely been drawn from studies on the regulation of body and organ size in one of the most diverse animal groups: the insects. Much of the morphological diversity seen across insect species is generated by changes in organ size and shape relative to the whole body (Shingleton et al., 2007, 2008). In holometabolous insects, body size is a function of the larval rearing environment, in which nutritional conditions play a major role. As adults have a sclerotized outer skeleton that prevents further growth, the adult body size is fixed once larvae stops feeding at the onset of metamorphosis. Moreover, many adult organs develop inside the larval body as imaginal discs and respond to the same cues that control

whole body growth. Therefore, like adult body size, the size of many adult organs is determined by the amount of growth that imaginal discs achieve during development.

Although significant progress has been made in understanding the hormonal mechanisms underlying nutritional plasticity of organ size in non-model insects (Beldade et al., 2011), recent advances in *D. melanogaster* have opened up unique opportunities to generate insight into the hormonal mechanisms through which nutrition changes organ size and produces novel and diverse morphologies. In *D. melanogaster*, like many holometabolous insects, three developmental hormones – the insulin-like peptides, juvenile hormone (JH), and the steroid moulting hormone ecdysone – translate signals from the nutritional environment to regulate body and organ growth (Mirth and Shingleton, 2012; Nijhout, 2003a). Although JH is a key regulator of growth in the tobacco hornworm *Manduca sexta* (Nijhout and Williams, 1974) and the dung beetles *Onthophagus taurus* (Emlen and Nijhout, 1999), its role in growth *D. melanogaster* was, until recently, controversial (Flatt, 2005; Mirth et al., 2014; Riddiford and Ashburner, 1991; Riddiford et al., 2010). In the following pages, I will therefore focus on what is known about the role of the *D. melanogaster* insulin-like peptides (dILPs), the insulin/insulin-like growth factor signalling (IIS) pathway, and the ecdysone signalling pathway in regulating nutritional plasticity in body and organ size.

1.4.1 Nutrition and the insulin/insulin-like growth factor signalling (IIS) pathway

In *D. melanogaster*, and many other animals, nutrition modifies body and organ growth through the action of the IIS pathway (Figure 1.2). In rich nutritional environments, neurosecretory cells in the brain, the insulin-producing cells, synthetize and secrete high amounts of dILPs. Only three

of the eight dILPs – dILP2, dILP3, and dILP5 – are exclusively expressed in the insulin-producing cells (Ikeya et al., 2002; Rulifson et al., 2002). The expression of these dILPs is nutrient dependent; starvation represses both their synthesis and secretion (Brogiolo et al., 2001; Ikeya et al., 2002). Further, ablation of the insulin-producing cells reduces adult body size in a similar fashion to starvation (Rulifson et al., 2002). These findings indicate that most of the nutrition-dependent growth is presumably regulated by the dILP production in the insulin-producing cells. The additional dILPs are expressed in several different tissues, including the imaginal discs, the mid gut, and the ventral nerve cord, and are thought to have systemic effects on growth (Brogiolo et al., 2001; Colombani et al., 2012; Garelli et al., 2012).

After being released into the insect bloodstream, dILPs act on target tissues by binding to the insulin receptor (InR) (Brogiolo et al., 2001). Once InR is activated, a highly conserved phospho-kinase signal transduction cascade, the IIS, is induced ultimately regulating cell growth and division. This is mainly achieved by activating positive growth regulators, such as the protein kinase Akt, and suppressing negative growth regulators, such as the transcription factor Forkhead Box class (FOXO) and the Tuberous Sclerosis Complex 1 and 2 (TSC1/2) (reviewed in (Taniguchi et al., 2006).

The suppression of TSC1/2 allows an additional nutrient-sensitive pathway, the target of rapamycin (TOR) signalling pathway, to remain active. The TOR pathway responds directly to intracellular amino acid concentrations via the TOR complex and regulates a number of cellular processes to promote growth (Gao et al., 2002; Sarbassov et al., 2005). In addition, the TOR complex itself regulates the IIS pathway by activating Akt (Sarbassov et al., 2005), which illustrates the extensive crosstalk between the two nutrition-sensitive pathways. Suppressing any component in the IIS pathway slows growth and results in smaller adults in a similar manner as starvation (Britton et al., 2002; Brogiolo et al., 2001). Combined, these findings illustrate that the circulating levels of dILPs and the IIS pathway coordinate growth rate with nutritional inputs.

Additionally, the IIS pathway also controls the length of the growth

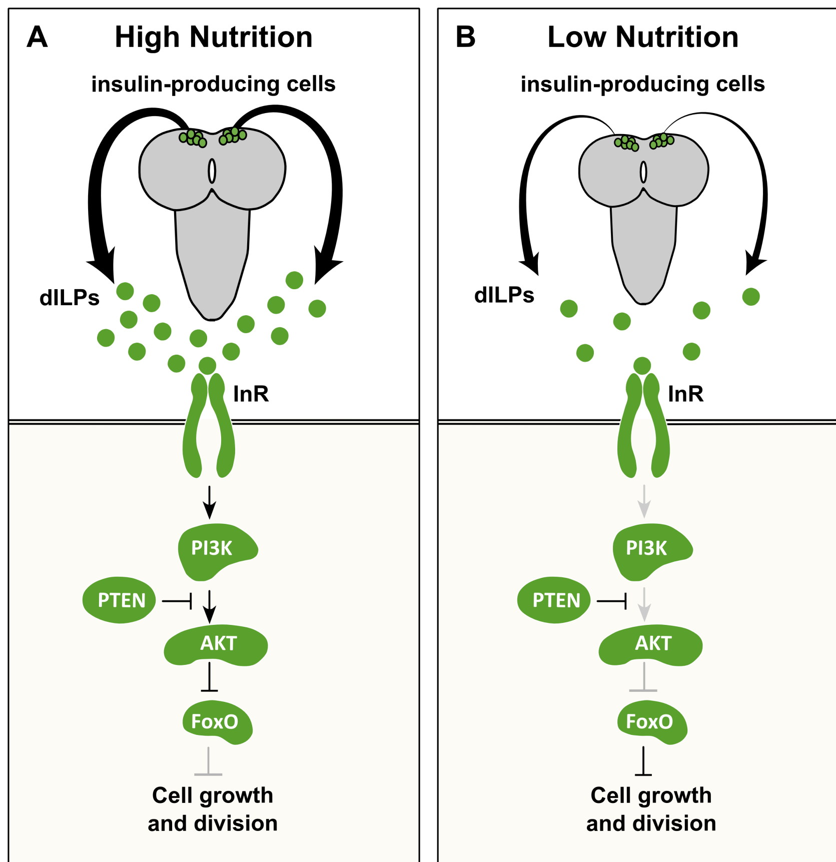

Figure 1.2: The IIS pathway in *D. melanogaster* The secretion of dILPs by the insulin-producing cells in the brain depends on nutrition. (A) Under high nutritional conditions, high levels of dILPs are expressed and activate the IIS pathway, promoting cell growth and division primarly by activating Akt and supressing the activitity of FOXO. (B) In contrast, when larvae are malnourished, low levels of dILPs are expressed, resulting in a reduced activity of IIS pathway, and consequently, reduced growth.

period – another crucial determinant of body and organ size in insects. The IIS pathway controls the growth period primarily by regulating the timing of the pulses of the steroid hormone ecdysone at specific stages in development (Koyama et al., 2014). How does ecdysone, in turn, regulate the duration of the growth period and ultimately body and organ size?

1.4.2 Nutrition and the ecdysone signalling pathway

Ecdysone is synthetized and secreted by the prothoracic glands in a series of discrete peaks throughout larval and pupal development. This periodic release of ecdysone together with a temporal- and tissue-specific expression of the ecdysone receptor complex, a heterodimer between Ecdysone Receptor (EcR) and Ultraspiracle (Usp), orchestrate many aspects of larval development: from larval molts and metamorphosis to growth and differentiation of target tissues (reviewed in (Yamanaka et al., 2013).

Several studies have uncovered that one small peak of ecdysone that occurs early in the third and final instar (L3) larvae is sensitive to nutritional conditions (Caldwell et al., 2005; Colombani et al., 2005; Koyama et al., 2014; Layalle et al., 2008; Mirth, 2005). This small peak of ecdysone reaches its maximum at around 8 to 10 h after third larval ecdysis (AL3E) (Koyama et al., 2014; Warren et al., 2006) and induces a key developmental transition, critical weight. Critical weight determines when to end growth and initiate metamorphosis, thereby regulating body and organ size (Koyama et al., 2014; Mirth and Riddiford, 2007; Mirth and Shingleton, 2012). Starving larvae before reaching critical weight significantly delays the timing of the ecdysone peak, which in turn, delays the onset of metamorphosis (Beadle et al., 1938; Mirth et al., 2005; Shingleton et al., 2005; Stieper et al., 2008) and delays patterning of the presumptive adult tissues, the imaginal discs (Mirth et al., 2009). Conversely, starvation after critical weight accelerates the onset of metamorphosis and no longer prevents continued patterning and growth of the imaginal discs in the absence of nutrition (Beadle et al., 1938; Mirth et al., 2005; Mirth et al., 2009; Shingleton et al., 2005).

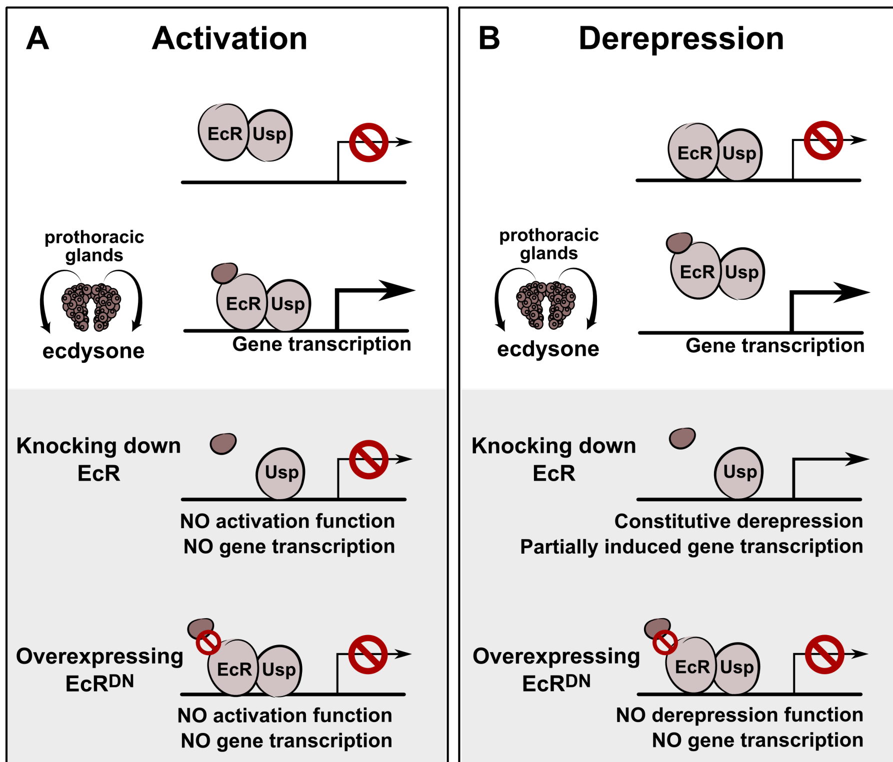

Figure 1.3: The activation and derepression functions of ecdysone signalling. Ecdysone binds to its receptor, a heterodimer between EcR and Usp, to induce two types of functions.(A) Activation function: ecdysone binds to EcR-Usp and directly activates gene transcription. (B) Derepression function: ecdysone binds to EcR-Usp and relieves the repressive action of the EcR-Usp, allowing gene transcription. Knocking down either EcR or Usp partially activates gene transcription, while overexpressing a dominant negative form of EcR with a mutated ligand-binding domain (EcR^{DN}) prevents gene transcription.

Ecdysone exerts its effects by binding to the EcR/Usp heterodimer complex. This complex represses the transcription of a subset of ecdysone target genes in the absence of ecdysone (Figure 1.3B) (Brown et al., 2006; Cherbas, 2003; Schubiger and Truman, 2000; Schubiger et al., 2005). Once ecdysone binds to EcR/Usp, it induces target gene transcription either by direct activation via EcR/Usp (Figure 1.3A) or by relieving the repressive action of the EcR/Usp (Figure 1.3B). Several genetic tools in *D. melanogaster* allow us to explore the specific roles of ecdysone signalling in body and organ growth. For instance, knocking down either EcR or

Usp, using RNAi, eliminates the repressive function of EcR/Usp, thereby partially inducing ecdysone function (Figure 1.3A) (Brown et al., 2006; Cherbas, 2003; Mirth et al., 2009; Schubiger et al., 2005). Conversely, overexpressing a dominant negative form of EcR with a mutated ligand-binding domain abolishes both the derepression and activation functions of ecdysone (Figure 1.3B)(Brown et al., 2006; Cherbas, 2003; Hu et al., 2003). In Chapter 3, I took advantage of these two well-described genetic tools to investigate the role of ecdysone signalling in regulating nutritional plasticity in organ size.

1.4.3 Organ-specific sensitivities to nutrition

If the levels of circulating dILPs reflect the nutritional status of an insect, how do different organs respond differentially to nutritional variation? As discussed above, the size of the male genitalia and the CNS is relatively invariant across nutritional conditions (Cheng et al., 2011; Shingleton et al., 2005; Tang et al., 2011). This low sensitivity to nutrition is achieved through different mechanisms. In the case of the CNS, InR-independent activation of the IIS pathway allows the CNS to maintain its growth rate even when circulating dILPs are low (Cheng et al., 2011). Alternatively, the male genitalia reduces its plasticity in response to nutrition by expressing low levels of *foxo* mRNA (Tang et al., 2011). When circulating dILPs and the activity of the IIS pathway are reduced, FOXO remains in the nucleus and supresses growth (Jünger et al., 2003). As the male genitalia expresses low levels of *foxo*, it is able to maintain its size even when larvae are malnourished (Figure 1.4A) (Shingleton et al., 2005, 2009; Tang et al., 2011). Overexpressing FOXO in the male genitalia increases its sensitivity to nutrition and results in smaller genitalia (Figure 1.4A) (Tang et al., 2011). Despite the differences in mechanisms between the CNS and the male genitalia, ultimately these organs are protected from the effects of poor nutrition by retaining high levels of activity of the IIS pathway irrespective of nutritional conditions.

Changing the level of activity of the IIS pathway in organs that scale proportionally with body size, as the wing discs, can result in

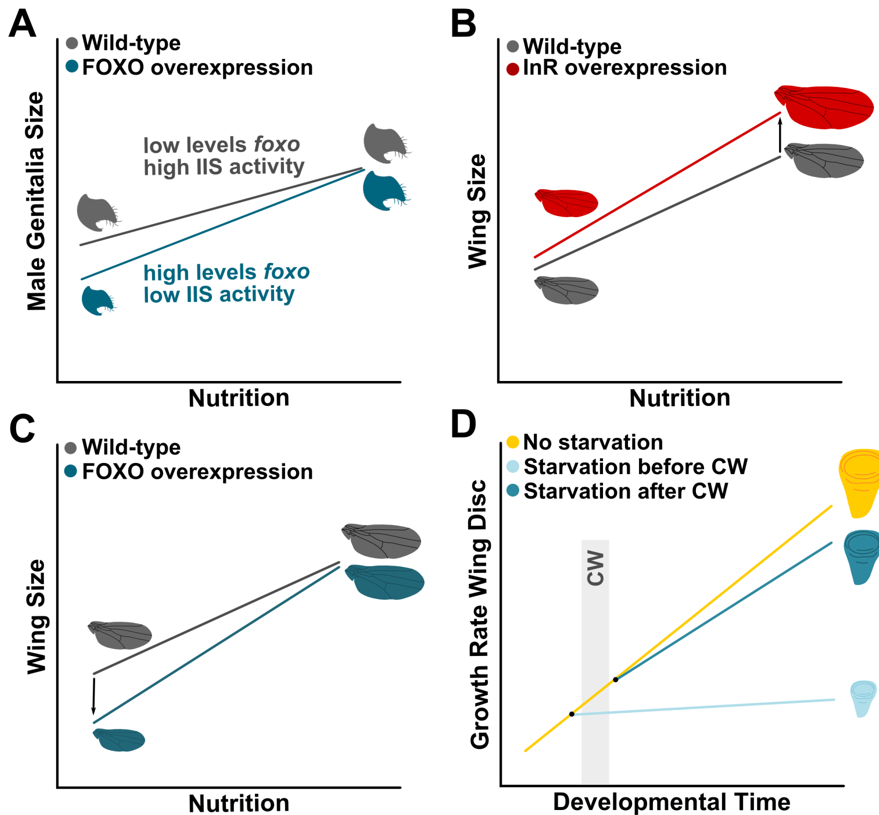

Figure 1.4: Organs differ in their sensitivity to nutrition. (A) The male genital disc maintains its size even when larvae are poorly fed. This reduction in nutritional sensitivity is achieved by reducing the levels of *foxo* mRNA and retaining high IIS activity in low nutritional environments (grey line). Overexpressing FOXO in the male genitalia increases its sensitivity to nutrition (blue line). (B, C) Nutrition affects the size of the wings in proportion with body size (grey line). (B) An increase of *InR* expression results in an increase in nutritional sensitivity by enhancing wing size in large flies (red line). (C) An increase of *foxo* expression enhances the nutritional sensitivity of the wing by supressing wing size in small individuals (blue line). (D) The sensitivity to nutrition of the wing discs varies with developmental time. Before critical weight, starvation severely reduces the growth of the wing disc. On the other hand, discs grow considerably even in post-critical weight larvae that are malnourished.CW:critical weight. Adapted from (Shingleton and Frankino, 2013; Shingleton and Tang, 2012; Shingleton et al., 2008)

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an exaggerated response to nutrition (Shingleton and Frankino, 2013; Shingleton and Tang, 2012). Overexpressing either FOXO or InR specifically in the wing disc increases its sensitivity to nutrition making it hyperallometric (i.e. disproportionally larger) in relation to body size (Figure 1.4B, C). However, this hyperallometry is achieved through different ways: increasing *InR* expression resulted in an exaggerated increase in the wing size of larger individuals, but has little or no effects in the wing size in smaller individuals (Figure 1.4B) (Shingleton and Tang, 2012). On the other hand, an increase in *foxo* expression led to a disproportionally small wing size in smaller individuals, but almost no effect in larger individuals (Figure 1.4C) (Shingleton and Tang, 2012). Thus it appears that organs can display exaggerated responses to nutrition by modulating the IIS pathway at several levels of its action (Shingleton and Frankino, 2013).

Organs can also change their sensitivity to nutrition with developmental time. For instance, starving pre-critical weight larvae compromises wing disc growth and differentiation, but after critical weight starvation has a more modest effect on the development of the wings discs; that is, discs grow considerably and continue to differentiate even when post-critical weight larvae are poorly fed (Figure 1.4D) (Mirth et al., 2009; Shingleton et al., 2008). This switch in sensitivity to nutrition at critical weight seems to be mediated by changes in the IIS pathway. Supressing the IIS pathway just after critical weigh abolishes body growth, but the wing discs continue to grow presumably until their size is appropriate for the much reduced body size (Figure 1.4D)(Shingleton et al., 2005, 2008). These findings have led some authors to hypothesize that an intrinsic growth rate that does not require nutritional inputs may enable further growth of the developing organs when nutrition, and accordingly the IIS, is severely reduced (Nijhout et al., 2014; Shingleton et al., 2008).

The activity of ecdysone signalling at critical weight may account for the switch in sensitivity to nutrition of developing organs. Knocking down EcR specifically in the wing discs of starved pre-critical weight larvae allows differentiation of the wing disc to proceed (Mirth et al., 2009). A similar observation was reported for the optic lobe of the *Drosophila* CNS

(Lanet et al., 2013). These findings suggest that ecdysone signalling acts in target tissues and allows their development to proceed even in the absence of nutritional inputs.

1.5 Aims and thesis scope

Even though developmental plasticity has gradually become a fundamental aspect in our evolutionary thinking, there are still many issues to be solved. To fully comprehend the role of plasticity in evolution, we first need to understand how developmental processes are regulated by environmental signals to generate a diverse range of phenotypes. Developmental plasticity should be seen as a fundamental source of phenotypic variation, a key condition for natural selection to operate. In this thesis, I therefore implemented a simple, yet powerful, developmental approach to investigate how the environment shapes the developmental trajectory of a developing organ to generate distinct morphologies. Specifically, the aim of this thesis was to investigate i) how developmental processes are modified by the environment, particularly nutrition, within a species and ii) compare whether similar developmental changes account for differences between species.

For this purpose, I used ovariole number, an important determinant of ovary size and female fecundity, in *Drosophila*. Ovariole number is determined during larval stages and is highly plastic in response to several environmental conditions, including nutrition and temperature. Moreover, ovariole number shows remarkable diversity among *Drosophila* species. Thus, ovariole number is an excellent model to address the aims of this thesis.

Despite the potential relevance of developmental windows of environmental sensitivity on the outcome of plasticity, few studies in insects have taken this factor in consideration when addressing the effects of nutrition in body and organ growth. This issue will be addressed in Chapter 2. Here I examined the existence of critical periods of sensitivity to nutrition during ovary development in *D. melanogaster*, with special emphasis whether critical weight separates distinct phases of nutritional

sensitivity. This will be the first step in elucidating which development processes during ovary development are modified by nutrition to generate differences in ovariole number.

The role of hormonal pathways in regulating nutritional plasticity in ovariole number will be explored in Chapter 3. Much of the current research has underscored hormonal signals as key regulators of developmental plasticity. However, how hormones regulate the nutritional sensitivity of a developing organ with developmental time has not been fully addressed. In this chapter, I asked whether IIS and/or ecdysone signalling pathways act at critical weight to regulate nutritional plasticity in ovariole number. I carefully characterized the contribution of each signalling pathway in the regulation of three developmental processes that I previously described to account for the nutritional-induced differences in ovariole number (Chapter 2). This powerful approach sheds an interesting light on the hormonal regulation of nutritional plasticity in ovariole number.

Chapter 4 will deal with the diversity in ovariole number between two subspecies of *D. mojavensis*. Developmental plasticity may facilitate phenotypic diversification among populations through the process of genetic accommodation. One prediction from this scenario is that the developmental processes that are responsible for the divergent morphologies between populations should to some degree resemble the developmental processes that initially emerged through developmental plasticity (Wund, 2012). I therefore characterized which of the developmental processes altered by nutrition underlie the differences in ovariole number between two subspecies of *D. mojavensis* that differ in ovariole number, *D. mojavensis sonorensis* and *D. mojavensis wrigleyi*. I further explored the degree of nutritional plasticity in the two subspecies by rearing them in four different food concentrations and analysing the effects on three traits: ovariole number, female body size and developmental time. Additionally, I examined ovariole number and female body size in F1 and F2 hybrids between the two subspecies as a preliminary approach to uncover the genetic basis for the evolutionary diversification in ovariole number.

Finally, in Chapter 5, I will discuss the main contributions of my thesis work and present future avenues of research that would further demonstrate that the environment not only acts as a selective agent, but it also contributes to the creation of novel and diverse life forms.

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2

NUTRITIONAL PLASTICITY IN OVARIOLE NUMBER IN *DROSOPHILA MELANOGASTER*

"A world of endless possibilities and infinite outcomes. Countless choices define our fate: each choice, each moment, a moment in the ripple of time. Enough ripple, and you change the tide... For the future is never truly set."

– from the film *X-Men: Days of Future Past* (2014)

Abstract

The extent to which an organism can adjust its developmental trajectory in response to environmental conditions, known as developmental plasticity, often depends on critical periods of environmental sensitivity. Here, I identify two phases of sensitivity to nutrition that regulate plasticity in ovariole number, an important determinant of fecundity and ovary size, in *Drosophila*. These two phases are separated by the developmental transition at critical weight. In the first highly-sensitive phase, optimal nutrition is required to promote ovary growth and to induce the onset of terminal filament cell (TFC) differentiation, which serves as a starting point for ovariole development. The second phase begins after TFC differentiation is initiated at critical weight. In this phase, the formation of terminal filaments (TFs) through intercalation of TFCs and ovary growth continue, albeit at reduced rates, in larvae that are poorly fed. These results shed a new light on how organs change their sensitivity to environmental variation at critical weight.

Publication

Chapter 2 and 3 are part of a manuscript submitted for publication, authored by C.C. Mendes and C.K. Mirth.

Authors' contributions

Christen Mirth and Cláudia Mendes conceived this study. Cláudia Mendes performed and analysed all experiments and wrote this chapter.

2.1 Introduction

Developmental plasticity, the ability of an organism to adjust its developmental trajectory in response to environmental variation, is a seemingly universal property of all multicellular organisms. Often, the extent of developmental plasticity depends not only on the traits and environmental conditions considered (Mirth and Shingleton, 2012), but also on the existence of phases of environmental sensitivity, commonly referred as critical periods, during which developmental processes can respond plastically (Koyama et al., 2013; Nijhout, 2003a). In the most extreme cases, an environmental cue within a critical period triggers a developmental switch between alternative developmental trajectories, giving rise to distinct phenotypes, such as dramatic seasonal differences in the pigmentation of butterfly wing patterns and different body sizes and shapes in honeybee castes (Brakefield et al., 1996; Smith et al., 2008). Understanding how developing organs change their sensitivity to environmental conditions, and how this influences their plastic response, is an important step towards a comprehensive knowledge of how the environment generates new phenotypic variants.

Nutritional status is one of the major regulators of body and organ growth and its effects have been extensively studied in insects, in particular, in the fruit fly, *Drosophila melanogaster*. In *D. melanogaster*, like many insects, nutrition regulates growth by regulating a key developmental transition, critical weight, which occurs around 10 h after moulting to the third and final instar larvae (L3) (Beadle et al., 1938; Koyama et al., 2014; Mirth et al., 2005; Nijhout and Williams, 1974; Shingleton et al., 2005; Stieper et al., 2008). Starving larvae before critical weight causes them to significantly delay the onset of metamorphosis (Beadle et al., 1938; Mirth et al., 2005; Shingleton et al., 2005; Stieper et al., 2008), whereas starving larvae after critical weight induces early metamorphosis (Beadle et al., 1938; Mirth et al., 2005; Stieper et al., 2008). As critical weight determines when to end growth and initiate metamorphosis, the underlying mechanisms regulating its attainment have been studied extensively (Beadle et al., 1938; Koyama et al., 2014; Mirth et al., 2005; Nijhout and Williams, 1974; Shingleton et al., 2005; Stieper et al., 2008).

Critical weight also regulates the sensitivity of developing organs to nutrition over developmental time. Larvae starved before reaching critical weight delay the patterning of their presumptive adult tissues, the imaginal discs (Mirth et al., 2009). Conversely, starvation after critical weight allows continued patterning and growth of the imaginal discs (Mirth et al., 2005, 2009; Shingleton et al., 2008). This additional role of critical weight has been overlooked in current research, and importantly, whether critical weight determines periods of nutritional sensitivity has not yet been fully investigated. In this chapter, I attempt to elucidate how developing organs change their sensitivity to nutrition over developmental time, with special emphasis on the potential role of critical weight in mediating nutritional sensitivity.

To address this issue, I used ovariole number in *D. melanogaster* as a model. Ovarioles are egg-producing structures in the insect ovary that directly affect female reproductive capacity (Boulétreau-Merle et al., 1982; R' kha et al., 1997; Klepsatel et al., 2013b,a). Although little is known about the genetic cascades involved in ovariole development (Cheng et al., 2011; Forbes et al., 1996; Godt and Laski, 1995; Patel et al., 1989; Sahut-Barnola et al., 1995; Sarikaya and Extavour, 2015), the cellular events mediating this process are better characterized. Ovariole development occurs during the third instar (L3) larval and early pupal stages (Kerkis, 1931; King, 1970; King et al., 1968) through the intercalation of terminal filament cells (TFCs) into stacks of seven to ten flattened cells, called terminal filaments (TFs) (Godt and Laski, 1995; Sahut-Barnola et al., 1995, 1996). Each TF defines the position of one ovariole and thus, the number of TFs at pupariation is equivalent to the number of ovarioles in the adult (Godt and Laski, 1995; Hodin and Riddiford, 1998; Sahut-Barnola et al., 1995; Sarikaya et al., 2012).

Nutritional conditions during larval stages regulate ovariole number (Bergland et al., 2008; Hodin and Riddiford, 2000; Sarikaya et al., 2012; Tu and Tatar, 2003). Previous studies of the developmental effects of nutrition on ovariole number have shown that diluting the food on which larvae were raised altered ovariole number by changing the total number of TFCs (Sarikaya et al., 2012) or the rate of TF formation in late L3 larvae (Hodin

and Riddiford, 1998). However, it was unclear whether ovary development exhibits critical periods of nutritional sensitivity, and importantly, how the developmental processes are modified by nutrition at different periods of sensitivity. I therefore examined whether changes in nutrition at specific stages during L3 larvae influence the plastic response of ovariole number. I further investigated how distinct stage-specific developmental processes during ovary development respond to changes in nutrition and account for nutritional-induced differences in ovariole number.

2.2 Materials and Methods

2.2.1 Fly stock

To assess the effects of larval nutrition on ovariole number, I used an outbred population (wild type) of *Drosophila melanogaster* established in the laboratory of Dr. Élio Sucena in 2007, originating from 160 fertilized females collected in Azeitão, Portugal (Martins et al., 2013). The population was kept in laboratory cages with high census (> 1500 individuals) and maintained at constant temperature (25°C) on standard fly food (4.5% molasses, 7.2% sugar, 7% cornmeal, 2% yeast extract, 1% agar and 2.5% Nipagin solution).

2.2.2 Larval staging and dietary manipulations

Adults were allowed to lay eggs for two to six hours on fresh food plates (60×15 mm Petri dish). Egg density was controlled to prevent overcrowding (approximately 200 eggs per plate). Larvae were selected 0-2 hours after ecdysis to L3 (AL3E) and transferred onto new food plates (40-60 larvae per plate) to feed until they reached the appropriate age. To determine critical periods of sensitivity to nutrition in ovariole number, 20-30 larvae of the appropriate age were transferred to vials containing either 20% sucrose on 0.5% agar medium (sucrose-only food) or standard fly food (standard food) until the end of the feeding period (Figure 2.1A). On sucrose-only food, most larvae survived until pupariation and adult

eclosion. To obtain L3 ovaries, larvae of the appropriate age were dissected and processed for immunocytochemistry (Figure 2.1C, 2.3A, 2.5A). All experiments were performed at 25°C.

2.2.3 Measurements of life-history traits: developmental time, female weight, early female fecundity and ovariole number

To determine the average time to pupariation, newly ecdysed L3 larvae were transferred to vials (20-30 larvae per vial) containing standard food. The number of larvae pupariating (immobile larvae with evaginated spiracles) was counted in 2 h intervals until all larvae pupariated. I used pharate weight as a proxy of adult body size (Mirth et al., 2005). Pharate adults were collected from food vials and food residuals were carefully cleaned off from the pupal cases using distilled water and a paintbrush. I distinguish females from males by the presence or absence of male-specific sex combs through the pupal case. Female pharate adults were individually weighed on a Sartorius SE2 ultramicrobalance.

To determine early fecundity, newly eclosed females were individually maintained in vials on standard food with one male of the same food/time point. Individuals were transferred to fresh vials every day during the first three days after eclosion. All eggs were counted daily. To count adult ovariole number, newly eclosed flies were maintained in vials (ten females and five males per vial) on standard food until the time of dissection (4-6 days after eclosion) (Figure 2.1A). Ovaries were dissected in cold phosphate buffered saline containing 1% Triton X-100 (PBT) and ovarioles were teased apart and counted under a dissecting microscope.

2.2.4 Immunocytochemistry

Female larvae were selected by the small size of their gonads located in the posterior third of the fat body. Larvae were dissected in cold phosphate buffered saline (PBS) and fixed in 4% formaldehyde in PBS for 30 minutes at room temperature. Larvae were then washed three times for 20 minutes

with PBT and blocked in 2% normal donkey serum in PBT for 30 minutes. Primary antibody incubation in mouse anti-Engrailed (Developmental Studies Hybridoma Bank 4D9, 1:40) diluted into 2% normal donkey serum in PBT was conducted overnight at 4°C. After washing three times for 20 minutes in PBT, larvae were incubated in the dark with goat anti-mouse Alexa 568 (Invitrogen, 1:200) and TRICT-Phalloidin (Sigma, 1:200) diluted into 2% normal donkey serum in PBT overnight at 4°C. Larvae were rinsed with PBT and ovaries were mounted on a poly-L-lysine-coated coverslip using Fluoromount-G (SouthernBiotech).

2.2.5 Image Acquisition and Analysis

Samples were imaged using a Zeiss LSM 510 Meta confocal microscope using a 40x 1.3NA oil objective lens. During confocal image acquisition, the detection parameters were adjusted to avoid under- or overexposed pixels, and images were acquired through the full thickness of the ovary at 1 µm. Images were processed and analysed using ImageJ (NIH) and Adobe Photoshop (Adobe Systems). For each time point/genotype/food treatment, terminal filament cells (TFC) were identified by Engrailed expression. Forming terminal filaments (TFs) were identified by the presence of TFC in stacks with the characteristic flattened cell morphology, and total number of forming TFs were counted. For ovary volume, the ImageJ Volumest plugin was used (Merzin, 2008).

2.2.6 Statistical Analysis

All experiments were replicated at least twice. The preliminary experiment described in Figure 2.1C, D was performed one time with small sample size. The distribution of residuals was tested for normality using Q-Q plots and the appropriate statistical test was applied. For multiple comparisons, ANOVAs were performed followed by Tukey's multiple comparison test to evaluate pairwise differences. Welch t-test (parametric) and Wilcoxon rank sum test (non-parametric) were used to test differences in mean values between two samples. To determine differences in the rate of TF formation and of ovary growth, slopes were compared using the

function 'sm.ancova' under 'sm' library. All data analyses and statistics were conducted using R v3.1.2 (R Development Core Team, 2014). Plots were made using GraphPad Prism v6 (GraphPad Software). *p*-values are indicated in the text and figures.

2.3 Results

2.3.1 Two phases of sensitivity to nutrition regulate the plastic response of ovariole number

To determine critical periods of sensitivity to nutrition in ovariole number, I fed L3 larvae either on standard food or on sucrose-only food at timed intervals starting between 0 h to 30 h AL3E until the end of the feeding period (Figure 2.1A). Larvae fed on sucrose-only food are starved of protein, lipids and other micronutrients present in yeast, yet show higher rates of survival than when starved completely. Overall, larvae transferred to sucrose-only food between 0 and 25 h AL3E showed a significant reduction in ovariole number when compared to the controls transferred to standard food (Figure 2.1B). In contrast, transferring larvae to sucrose-only food at 30 h AL3E did not cause a significant reduction in ovariole number (Figure 2.1B).

Interestingly, the effect of the sucrose-only food in ovariole number depended on the timing at which larvae were transferred to the sucrose-only food (Figure 2.1B). To test for a significant change in the response to sucrose-only food over time, I applied a bi-segmental linear regression model to the data and tested for a significant change in slope. The relationship between ovariole number and the age at transfer to sucrose-only food (in h AL3E) has a significant change in slope around a single breakpoint (Davies' test for a change in slope, $p<0.0001$) at 11.5 h AL3E (95% CI: 9.37–13.64 h AL3E) (Davies, 1987; Muggeo, 2003, 2007). This estimated breakpoint coincides with the attainment of critical weight, suggesting that pre-critical weight ovaries are more sensitive to changes in nutrition than post-critical weight ovaries.

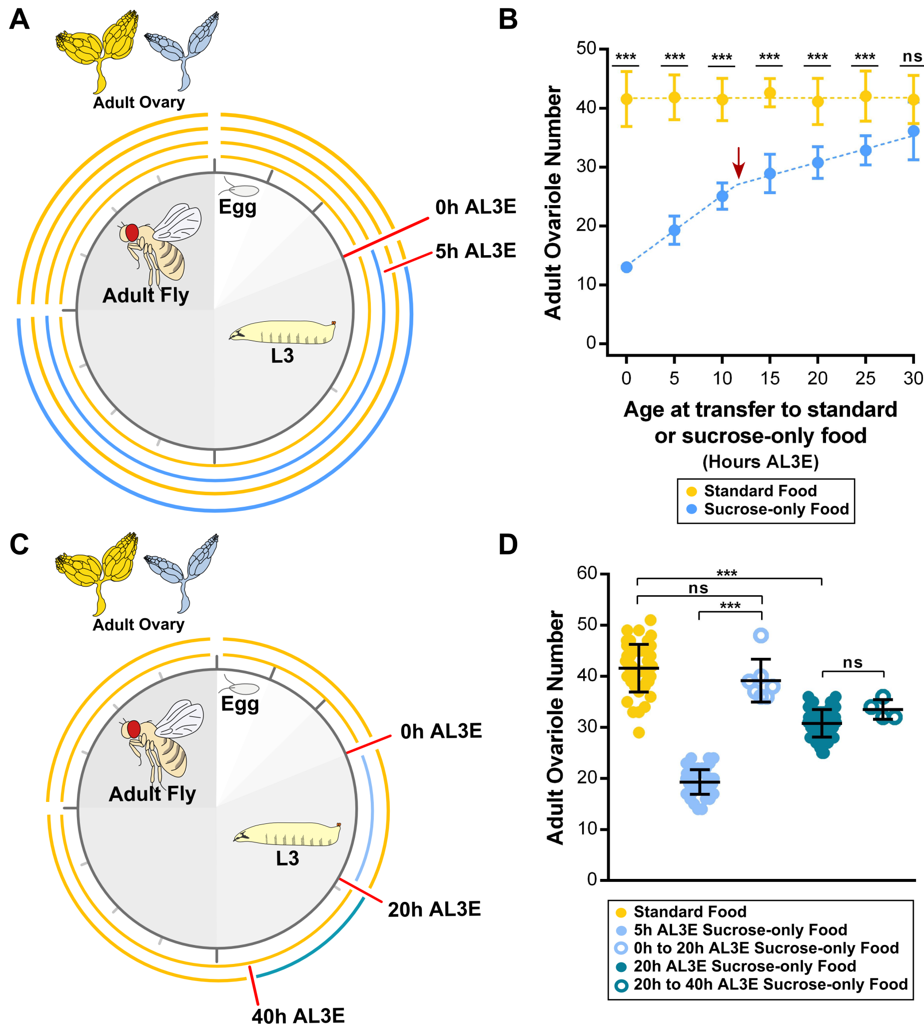


Figure 2.1: Changes in nutrition during the first phase of sensitivity have greater effects in ovariole number than in the second phase of sensitivity. (A) Experimental design to determine critical periods of sensitivity to nutrition in ovariole number. Only the first two time points are shown (0 h and 5 h AL3E). (B) Adult ovariole number from larvae transferred either to standard food (yellow circles) or to sucrose-only food (blue circles). Dashed lines show the best fitted-lines from the segmental regression analyses. n≥30 for all treatments. (C) Experimental design to determine whether the length of exposure to sucrose-only food influences ovariole number. (D) Adult ovariole number from larvae fed on standard food (yellow circles); larvae transferred to sucrose-only food either at 5 h AL3E (light blue circles) or at 20 h AL3E (dark blue circles) and larvae fed on sucrose-only food for a 20 h interval either between 0 h to 20 h AL3E (open blue circles) or between 20 h to 40 h AL3E (open dark circles). Plotted values represent means and error bars show 95% confidence intervals of means. Two-way ANOVA using Tukey's test: *p<0.05, ***p<0.001, ns non-significant. L3: third instar larvae; AL3E: after L3 ecdysis.

Nevertheless, the effects of the sucrose-only food in ovariole number could also be a direct consequence of different lengths of exposure to the sucrose-only food. To test this hypothesis, I performed a preliminary experiment where L3 larvae were fed on sucrose-only food for 20 h starting either at 0 h AL3E or at 20 h AL3E and then returned to standard food until the end of the feeding period (Figure 2.1C). As described above, ovariole number is severely reduced when larvae were transferred to sucrose-only food at 5 h AL3E (Figure 2.1B, D). Surprisingly, ovariole number in larvae fed on sucrose alone for a short period between 0 h to 20 h AL3E was similar to standard food control (Figure 2.1D). In contrast, when larvae were fed on sucrose-only food from 20 h to 40 h AL3E, ovariole number was significantly reduced (Figure 2.1D). This reduction in ovariole number was similar when compared to larvae transferred to sucrose alone at 20 h AL3E until the end of development (Figure 2.1B, D). These observations corroborate a previous study where re-feeding pre-critical weight larvae after a brief period of starvation delays pupariation for longer than the length of the starvation period, but does not affect final body size. After critical weight, when the duration of the larval growth period is fixed, short periods of starvation have no effect on the timing of pupariation and thus, larvae are unable to reach their optimal body size even after re-feeding (Beadle et al., 1938).

Ovariole number is positively correlated with egg production rate and therefore is closely related to fitness (Boulétreau-Merle et al., 1982; R' kha et al., 1997; Klepsatel et al., 2013b,a). As expected, I found that females that were fed on sucrose-only food as larvae at timed intervals starting between 5 h to 25 h AL3E until the end of the feeding period laid fewer eggs in the first three days after eclosion relative to standard food control (Figure 2.2). When I plotted ovariole number against daily egg production, I found that differences in ovariole number correlated with differences in the number of eggs laid (Figure 2.2). Thus, these results confirm that ovariole number is a good proxy for early female fecundity in *D. melanogaster*.

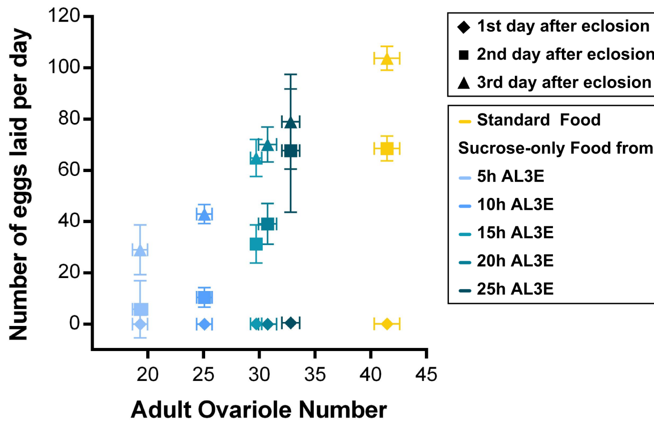


Figure 2.2: Ovariole number is positively correlated with early female fecundity. Number of eggs laid was counted in the first three days after eclosion (diamond: 1st day after eclosion; square: 2nd day after eclosion; circle: 3rd day after eclosion) from females fed on standard food as larvae (yellow symbols) and females fed on sucrose-only food as larvae at timed intervals starting between 5 h to 25 h AL3E (symbols with different shades of blue) until the end of the feeding period. Plotted values represent means and error bars show 95% confidence intervals of means. L3: third instar larvae; AL3E: after L3 ecdysis.

2.3.2 Ovary development during L3 larval stages

To further understand how nutrition regulates ovariole number, I first analysed ovary development in L3 larvae raised in standard food. When TFCs differentiate from the surrounding ovarian somatic cells, they upregulate expression of the transcription factor Engrailed (En) (Patel et al., 1989). Thus, I used En as a marker for TFC differentiation and TF formation.

Consistent with previous studies, TFCs were not observed in pre-critical weight ovaries (from 0-10 h AL3E) (Figure 2.3A, B) (Godt and Laski, 1995). At 15h AL3E, TFCs appeared in the medial side of the ovary and few short TFs were visible (Figure 2.3A, B). New TFCs continued to emerge from the surrounding ovarian somatic cells and gradually intercalated into forming TFs. The formation of new TFCs occurs in a lateral direction (Figure 2.3A, B) (Godt and Laski, 1995; Sahut-Barnola et al., 1995, 1996). At the end of L3, all of the approximately 18-22 TFs

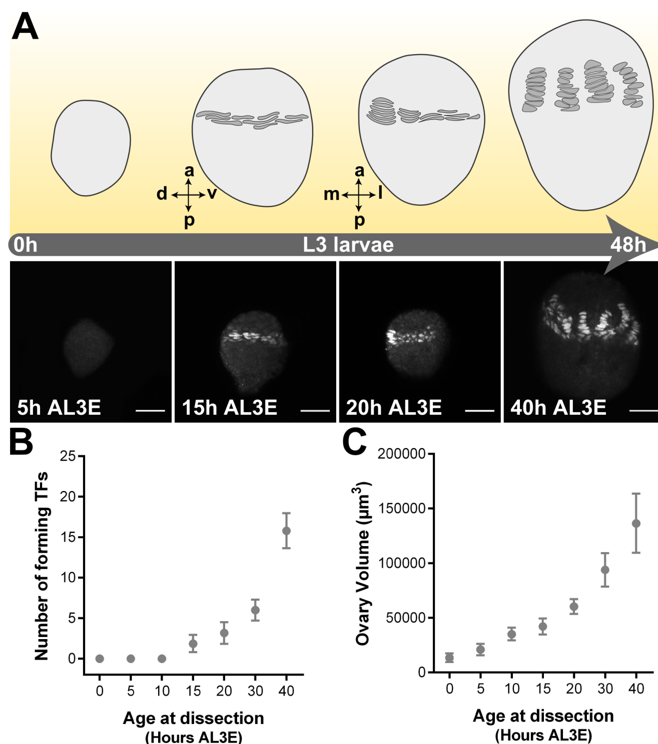


Figure 2.3: Ovary development during L3 larval stages under optimal nutritional conditions.(A) Schematic drawings representing ovary development in L3 larvae reared in standard food. Terminal filaments (TFs) are represented as dark grey symbols. Axis are presented as A-P, anterior-posterior; D-V, dorsal-ventral; M-L, medial-lateral. Pictures show developing ovary during L3 larval stages under standard food. Engrailed (grey) marks terminal filament cells (TFCs). Scale bar: 20µm. (B) Number of forming terminal filaments (TFs). (C) Ovary volume. Plotted values represent means and error bars show 95% confidence intervals of means. L3: third instar larvae; AL3E: after L3 ecdysis.

have formed (Figure 2.3A, B) (Godt and Laski, 1995; Hodin and Riddiford, 1998; Sarikaya et al., 2012). To assess the dynamics of ovary growth, I measured ovary volume over development time. Ovary volume increased exponentially throughout L3 larval development (Figure 2.3C), confirming results previously found in (Kerkis, 1931).

2.3.3 TF formation and ovary growth respond differently to pre- and post-critical weight nutrition

From my description of ovary development during L3 larval stages, I hypothesized that larval nutrition regulates one or all of the three developmental processes: i) the onset of differentiation of the first TFCs, representing the first step in ovariole development, ii) the rate at which new TFs emerged through intercalation of TFCs (referred as the rate of TF formation), and iii) the rate of ovary growth.

To test which of these processes respond to changes in nutrition, I fed larvae on sucrose-only food for 24 h, starting at 5 h intervals between 0 h to 25 h AL3E, and quantified the number of TFs and ovary volume for each condition at the end of this one-day starvation period (Figure 2.4A). When larvae were fed on sucrose-only food before reaching critical weight (before 10 h AL3E), I failed to observe any En-positive cells in the ovaries, indicating that the onset of TFC differentiation is delayed (Figure 2.4B-E, N). Wing discs and central nervous systems of larvae staged before 10 h AL3E did show En expression, indicating that this antigen was detectable in other tissues (Supplementary Figure S2.1). In addition, the ovary volume was severely reduced in larvae fed on sucrose-only food before 10 h AL3E (Figure 2.4O). In contrast, when larvae were transferred to sucrose-only food around the time of the critical weight transition (at 10 h AL3E), the majority of ovaries had few TFCs (Figure 2.4F, G) and in some ovaries TFCs were organized into short TFs (Figure 2.4N). Ovary volume was still greatly reduced in these larvae (Figure 2.4O). Finally, ovaries from larvae transferred to sucrose-only food after reaching critical weight (after 15 h AL3E), all had forming TFs (Figure 2.4H-M). Nevertheless, both TF number and ovary volume were moderately reduced when compared with larvae fed on standard food (Figure 2.4N, O).

Ovaries from pre-critical weight larvae fed on sucrose-only food for 24 h did not contain any TFCs (Figure 2.4B-E, N). However, these larvae did eventually give rise to adults with functional ovaries (Figure 2.1B and 2.2). Pre-critical weight larvae significantly delay the onset of pupariation and give rise to smaller adults when fed on sucrose-only food (Supplementary

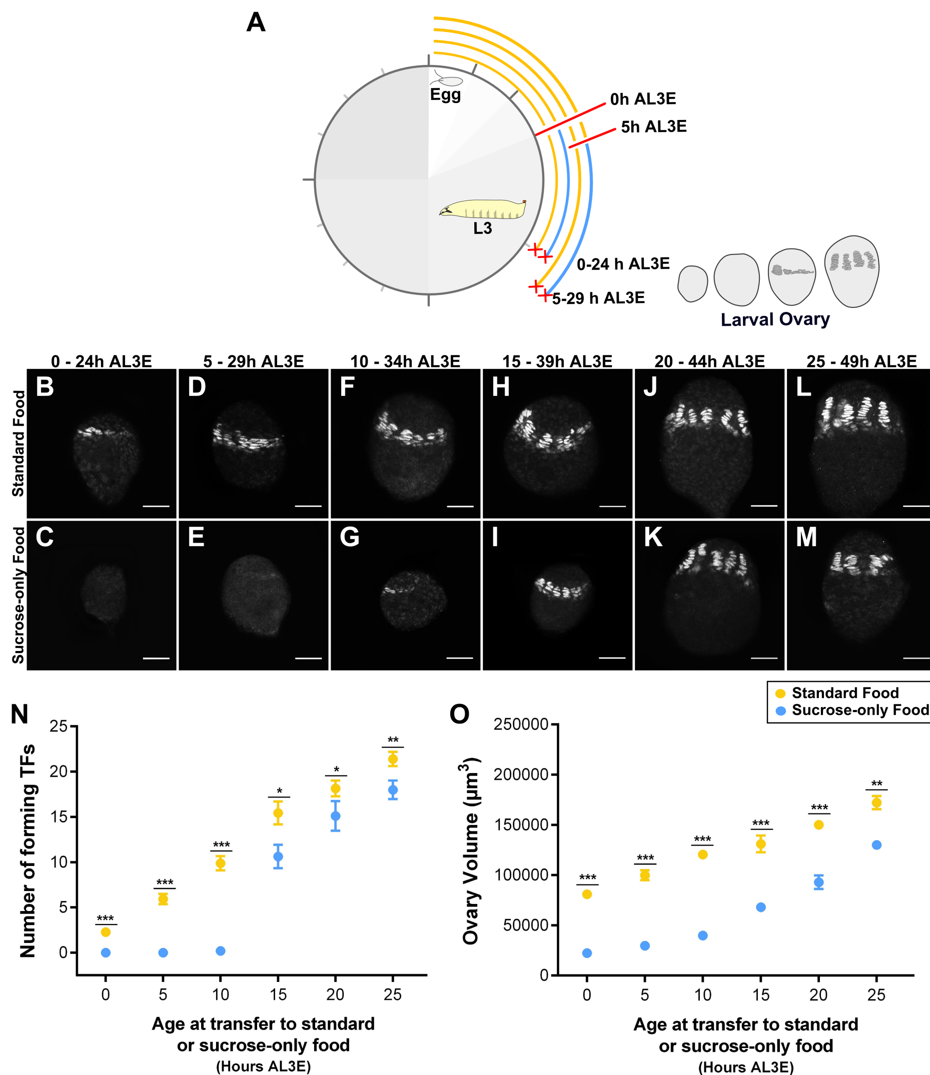


Figure 2.4: Distinct stage-specific developmental processes during ovary development are regulated by nutrition.((A) Experimental design to examine how developmental processes respond to changes in nutrition during L3 larval stages. Only the first two time points are shown (0 h and 5 h AL3E). Dissection times are marked with red crosses. (B-M) Shown is terminal filaments (TFs) marked with Engrailed (grey) in ovaries from larvae fed on (B, D, F, H, J, L) standard food or (C, E, G, I, K, M) sucrose-only food for 24 h starting between 0 h to 25 h AL3E. Scale bar: 20µm. (N) Number of forming terminal filaments (TFs) and (O) ovary volume of ovaries from larvae fed on standard food (yellow circles) or sucrose-only food (blue circles). Plotted values represent means and error bars show 95% confidence intervals of means. In some cases, error bars are too small to be shown. Welch Two sample t-test using Holm's *p-value* adjustment: *$p<0.05$, **$p<0.01$, ***$p<0.001$). L3: third instar larvae; AL3E: after L3 ecdysis.

Figure S2.2A). Therefore, I presumed that TF formation eventually occurs in these ovaries, even if its onset is delayed. To test this hypothesis, I fed pre-critical weight larvae on sucrose-only food and dissected the larval ovaries at three different time points (Figure 2.5A). Indeed, TFCs and few short TFs were observed at 49 h AL3E (Figure 2.5C-C´, E) and new TFs were still forming at 69 h AL3E, albeit at a significantly reduced rate (Figure 2.5E). A slower rate of TF formation relative to standard food controls was also found in ovaries from post-critical weight larvae fed on sucrose-only food (Figure 2.5D-D', E). These post-critical weight larvae pupariate at the same time as the standard food controls, but are smaller in body size (Supplementary Figure S2.2B). Furthermore, while ovaries from post-critical weight larvae fed on sucrose-only food showed a slight increase in ovary volume over L3 larval stages, feeding on sucrose-only food strongly arrested ovary growth in pre-critical weight larvae (Figure 2.5F).

2.4 Discussion

An important step towards a better understanding how the environment modifies the developmental trajectory of an organism to produce distinct phenotypes is to determine critical periods of environmental sensitivity. The present work identified such critical periods in the developing fly ovary. With my detailed characterization of the effects of nutrition on ovary development, I identified two phases of sensitivity to nutrition during L3 larval stage that regulate the plastic response of ovariole number. This switch in sensitivity coincides with the timing of critical weight (Koyama et al., 2014; Mirth et al., 2005, 2009; Shingleton et al., 2005). I further found that distinct developmental processes during ovary development respond differentially to changes in nutrition in each phase of sensitivity (Figure 2.6).

I have shown that the onset of TFC differentiation, which is induced around the timing of critical weight, is highly sensitive to nutritional inputs. Ovaries from pre-critical weight larvae fed on sucrose alone strongly delayed the onset of TFC differentiation. This result is in contrast with a previous report where larvae raised from egg to adult eclosion

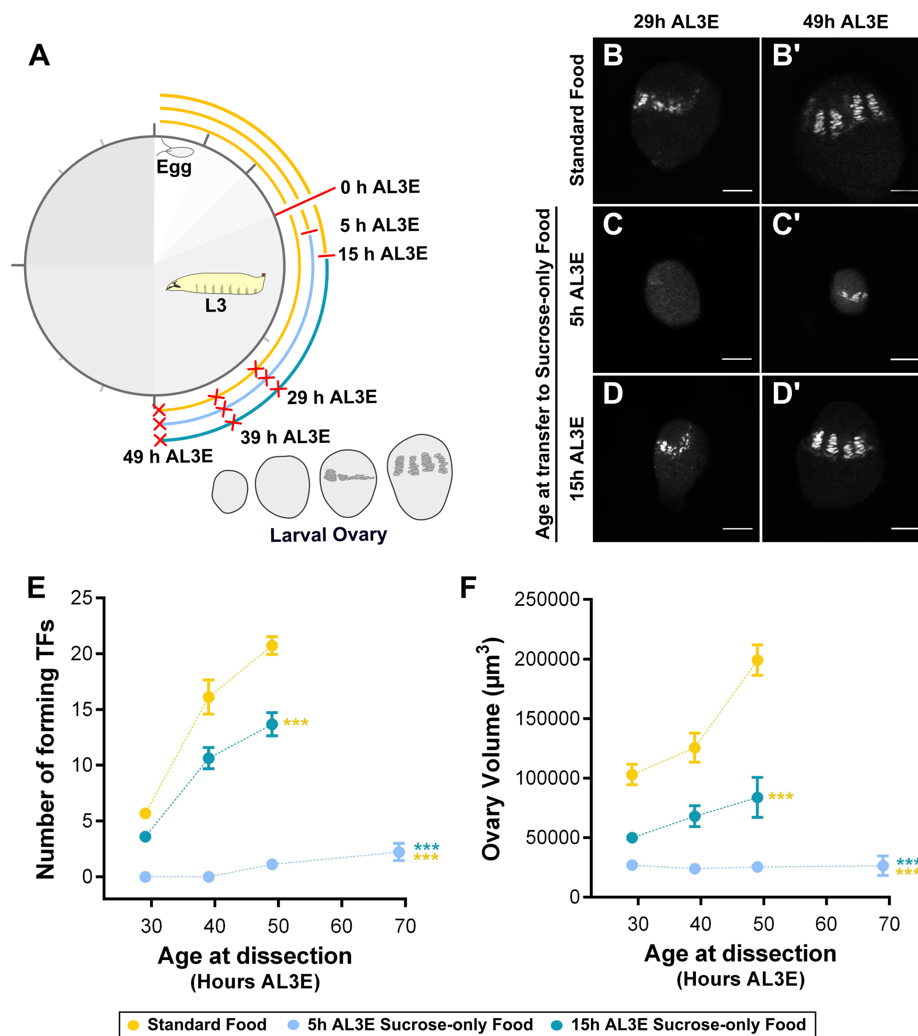


Figure 2.5: TF formation and ovary growth respond differently to pre- and post-critical weight nutrition.(A) Experimental design to examine the dynamics of TF formation and ovary growth in larvae transferred to standard food (yellow line) and in larvae transferred to sucrose-only food either at 5 h AL3E (light blue line) or at 15 h AL3E (dark blue line). Dissection times are marked with red crosses. (B-D') Shown is terminal filaments (TFs) marked with En (grey). (B-B') Ovaries from larvae reared on standard food. (C-D') Ovaries from larvae transferred to sucrose-only food from: (C-C') 5 h or (D-D") 5 h AL3E. Larvae dissected at (B, C, D) 29 h or (B´, C', D') 49 h AL3E. Scale bar: 20µm. (E) Number of forming terminal filaments (TFs) and (F) ovary volume of ovaries from larvae fed on standard food (yellow circles or transferred to sucrose-only food either at 5 h AL3E (light blue circles) or at 15 h AL3E (dark blue points). Plotted values represent means and error bars show 95% confidence intervals of means. In some cases, error bars are too small to be seen. ANCOVAs using Holm's *p-value* adjustment: ***p<0.001. L3: third instar larvae; AL3E: after L3 ecdysis.

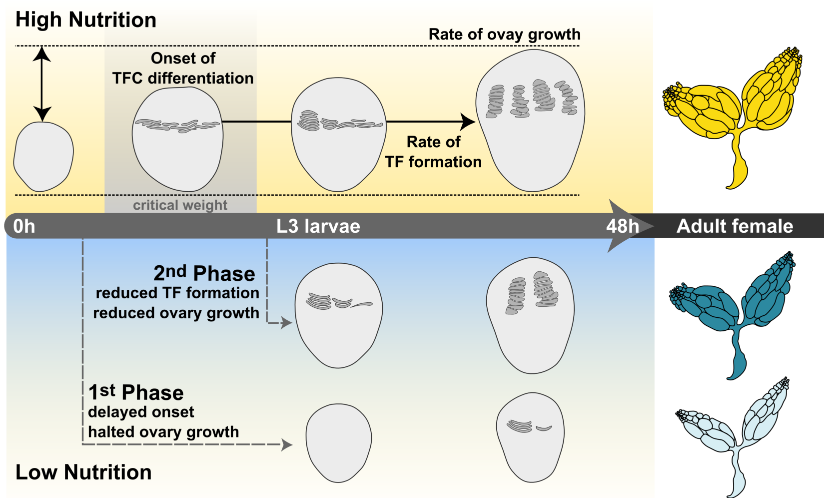


Figure 2.6: Critical weight separates two phases of sensitivity to nutrition in ovariole number. In the first, highly sensitive phase, poor nutrition arrests ovary growth and strongly suppresses the onset of terminal filament (TF) formation. Once TF formation is initiated around critical weight, TF formation and ovary growth proceed, although at a slower rate, in response to poor nutrition. These two phases of sensitivity influence the plastic response of ovariole number; changes in nutrition during the first phase of sensitivity have greater effects in ovariole number than in the second phase of sensitivity.

in food diluted to 50% its original concentration reduce ovariole number through changes in the rate of TF formation only at later L3 larval stages (Hodin and Riddiford, 2000). Differences in the timing of critical weight may explain these disparate results. Starvation before critical weight is attained delays the onset of pupariation (Beadle et al., 1938; Koyama et al., 2014; Mirth et al., 2005; Nijhout and Williams, 1974; Shingleton et al., 2005; Stieper et al., 2008). Hodin and Riddiford (2000) reported no differences in development time or body size between larvae raised on a food with half the nutrient content and well-fed controls (Hodin and Riddiford, 2000). Mostly likely, larvae reach critical weight at the same time in these two foods and, thus, the onset of TFC differentiation was not delayed. Conversely, I have shown that pre-critical weight larvae fed on sucrose-only food significantly delay the onset of pupariation and reduce their body size, suggesting that critical weight was delayed. These results imply that alterations in the timing of the onset of TFC differentiation

may only occur when the timing of critical weight is also affected. Thus, the attainment of critical weight and the onset of TF formation appear to be intimately connected and both are critical steps in regulating the plastic response of ovariole number.

Interestingly, I found that changes in nutrition during the second phase of sensitivity did not supress the formation of new TFs. In fact, new TFs continue to form in malnourished larvae. This suggests that while the initial trigger for TFC differentiation is highly sensitive to nutrition, subsequent TFCs continue to differentiate from the pool of TFC precursors irrespective of the nutritional conditions. Several other tissues also exhibit a similar switch in sensitivity to nutrition that allows progression of cell differentiation when larvae are poorly fed (Lanet et al., 2013; Mirth et al., 2009). For instance, after critical weight, neuroblasts in the optic lobe are able to generate the full repertoire of neuronal types independently of nutritional variation (Lanet et al., 2013). The attainment of critical weight may serve as a signal that ensures that sufficient endogenous nutritional reserves exist to sustain neuronal diversity (Lanet and Maurange, 2014). A similar mechanism may be employed to promote TFC differentiation under conditions of poor nutrition.

Even though TF formation proceeded under poor nutritional conditions, the rate at which new TFs were formed was significantly slower than standard food controls, resulting in a reduced number of TFs. Such reduction in the rate of TF formation alludes to changes in the production of new TFC precursors. However, our current knowledge on when and how TFC precursors are produced have thus far been limited (Lengil et al., 2015; Sahut-Barnola et al., 1996). Future work on identifying additional TFC markers may help us understand whether changes in nutrition during L3 larval stages affect the production of TFC precursors and how this may influence the rate of TF formation.

I additionally found that ovary growth is differentially affected in the two phases of sensitivity. In the first, highly-sensitive phase, poor nutrition precludes ovary growth. In contrast, during the second phase of sensitivity, poor nutrition reduces but does not prevent ovary growth. At the mechanistic level, we understand the most about how nutrition

modifies the growth trajectories of the wing imaginal discs (Garcia-Bellido and Merriam, 1971; Martin, 1982; Bryant and Levinson, 1985). After attainment of critical weight, wing discs have an intrinsic growth rate that promotes considerable growth under poor nutritional conditions. This intrinsic growth is not present before critical weight, and thus, wing discs arrest growth when pre-critical weight larvae are poorly fed (Shingleton et al., 2008). In light of these observations, I propose that developing ovaries may also have an intrinsic growth rate after critical weight that allows progression of growth in poorly-fed larvae.

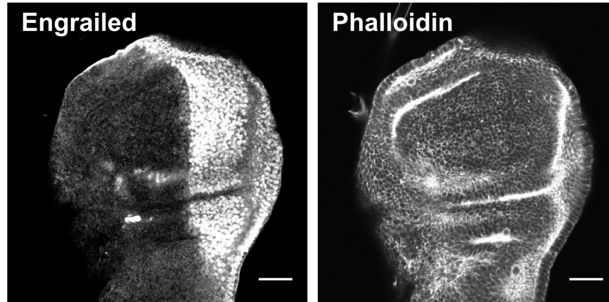
2.5 Conclusions

The results described in this chapter revealed that critical weight plays a fundamental role in reprograming the developing ovary's response to nutrition. Furthermore, this work contributes to a better understanding of the developmental processes that regulate ovariole number, and provides the developmental tools that will be used throughout this thesis.

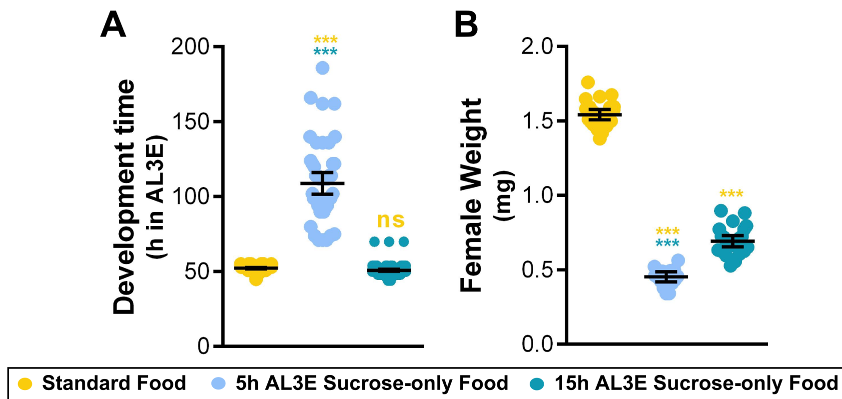
Acknowledgements

I would like to thank Vítor Faria and Nelson Martins for taking care of the outbred populations of *D. melanogaster*. Christen Mirth for teaching me how to count ovarioles and for commenting and proofreading this chapter. Élio Sucena for discussions throughout this project and for comments on an early version of this chapter. The Unit of Imaging at IGC for all the assistance during image acquisition and analysis. The Fly Facility, in particular Liliana Vieira, for making fly food every week.

Supplementary Figures



S 2.1: Engrailed is expressed in the wing discs of larvae reared in sucrose-only food. To confirm that the absence of TFCs (Engrailed-positive cells) was due to the diet manipulation and not to the immunocytochemistry protocol, I dissected wing discs or central nervous system (CNS) and analysed them together with the larval ovaries. Engrailed is expressed in the posterior compartment of the wing disc and in the neuroblasts in the CNS. Shown is a wing disc of larvae fed on sucrose-only food from 5 h AL3E expressing Engrailed. Phalloidin marks F-actin to outline cell membranes. Scale bar: 20µm. L3: third instar larvae; AL3E: after L3 ecdysis.



S 2.2: Larval nutrition affects development time and female weight.((A) Development time represented in hours after third instar ecdysis (h AL3E) to pupariation. Wilcoxon rank test using Holm's *p*-value adjustment: ***p<0.001, ns non-significant. (B) Female weigh. Welch Two sample t-test using Holm's *p*-value adjustment: ***p<0.001. Larvae fed on standard food (yellow circles) and larvae transfer to sucrose-only food either at 5h AL3E (light blue circles) or at 15h AL3E (dark blue circles). Plotted values represent means and error bars show 95 confidence intervals of means. L3: third instar larvae; AL3E: after L3 ecdysis.

3

HORMONAL SIGNALLING REGULATES PLASTICITY IN OVARIOLE NUMBER IN *DROSOPHILA MELANOGASTER*

"I mean, how could they know that because of their little dance the world lives? But it does. By simply doing what they're designed to do, something large and magnificent happens."

– from the film *Adaptation* (2002)

Abstract

Hormones coordinate body and organ growth with environmental conditions. Several studies have uncovered that changes in the timing and amount of hormone production are associated to numerous plastic responses in morphology, behaviour and physiology. However, how hormones act at specific stages in development to modify the sensitivity of a developing organ to environmental variation is poorly understood. In the previous chapter, I have shown that critical weight separates two phases of sensitivity to larval nutrition in ovariole number. Here, I set out to test whether two hormonal signalling pathways – the insulin/insulin-like growth factor signalling (IIS) and ecdysone signalling pathways – act at critical weight to control the response of the developing ovary to nutritional conditions and thus, regulate the plastic response of ovariole number. Indeed, I found that both IIS and ecdysone signalling pathways change the developing ovary's sensitivity to nutrition by regulating distinct stage-specific developmental processes that I have shown to be modified by nutritional conditions. These results enhance our understanding of the stage-specific action of hormones in regulating plastic responses.

Publication

Chapter 2 and 3 are part of a manuscript submitted for publication, authored by C.C. Mendes and C.K. Mirth.

Authors' contributions

Christen Mirth and Cláudia Mendes conceived this study. Cláudia Mendes performed and analysed all experiments and wrote this chapter.

3.1 Introduction

Environmentally-induced phenotypes are often induced by hormone signals that integrate cues from the external environment and coordinate developmental processes throughout the whole organism. This integration of environmental information is often reflected in changes in the timing and/or amount of hormone production during specific stages in development. These changes in hormone production can then affect the growth and differentiation of developing tissues and produce a wide range of distinct morphologies (Beldade et al., 2011; Koyama et al., 2013; Nijhout, 2003a). Moreover, changes in the activity of hormonal pathways have been shown to underlie differences in nutritional sensitivity between different organs (Tang et al., 2011). However, there is still a fundamental gap in our understanding of how hormones act at specific stages in development to change the sensitivity of a developing organ to environmental variation.

In the previous chapter, I have shown that a key developmental transition, critical weight, appears to reprogram the sensitivity of the developing ovary to nutritional conditions (see Chapter 2). While starving larvae before critical weight strongly compromises terminal filament cell (TFC) differentiation and ovary growth, starvation after critical weight has a more modest effect on ovary development; that is, ovary growth and the formation of new TFs continue, although at slower rates, in post-critical weight larvae that are poorly fed. Such changes in nutritional sensitivity greatly influence the plastic response of ovariole number. Interestingly, wing disc development shows a similar change in nutritional sensitivity at critical weight (Mirth et al., 2009; Shingleton et al., 2008). This switch in the nutritional sensitivity of the wing is regulated by two hormonal pathways, the insulin/insulin-like growth factor signalling (IIS) and ecdysone signalling pathways.

At critical weight, the prothoracic glands produce a small peak of ecdysone (Koyama et al., 2014). Once released, ecdysone binds to its receptor, a heterodimer between Ecdysone Receptor (EcR) and Ultraspiracle (Usp), in the wing discs and activates the transcription of target genes, allowing

the progression of differentiation to continue even in the absence of nutrition (Mirth et al., 2009). In addition, the growth of wing discs becomes less sensitive to nutrition after critical weight (Shingleton et al., 2008). Because IIS regulates body and organ growth in response to nutrition, the pulse of ecdysone at critical weight is thought to make the growth of the disc less sensitive to IIS (Koyama et al., 2013; Stieper et al., 2008).

Interestingly, both IIS and ecdysone signalling pathways regulate ovariole number (Gancz and Gilboa, 2013; Gancz et al., 2011; Green and Extavour, 2012, 2014; Hodin and Riddiford, 1998), and IIS is known to underlie the plastic response of ovariole number to larval nutrition (Green and Extavour, 2014). These studies reported that manipulating IIS in the larval ovary affects ovary volume and total TF number, but has no effect on the timing of the onset of TFC differentiation (Gancz and Gilboa, 2013; Green and Extavour, 2012, 2014). Repressing ecdysone signalling in the developing ovary delays TFC differentiation and reduces ovary volume (Gancz et al., 2011; Hodin and Riddiford, 1998). However, these studies have not explored the effects of IIS and ecdysone signalling pathways with sufficient temporal resolution to dissociate their roles in regulating TFC differentiation, the rate at which new TFs are formed, or the rate of ovary growth. Furthermore, the relative roles of IIS and ecdysone signalling pathways in altering developmental processes in response to nutrition have not been explored.

Here, I investigated whether IIS and ecdysone signalling pathways act during critical weight to regulate the developing ovary's sensitivity to nutritional conditions, and ultimately, influence the plastic response of the ovary. I first examined the relative contributions of each signalling pathway in regulating three developmental processes during ovary development: the onset of TFC differentiation, the rate of TF formation, and the rate of ovary growth in well-fed larvae. I next explored whether the nutritional sensitivity of these developmental processes is mediated by IIS and ecdysone signalling pathway.

3.2 Material and Methods

3.2.1 Fly Stocks

To genetically manipulate IIS and/or ecdysone signalling pathways, I used *traffic jam*-GAL4 (tj) [yw;PGawBNP1624/CyO; a gift of Lilach Gilboa] to drive expression in somatic cells of the larval ovary (Gancz et al., 2011). This line was crossed to w^{1118} (used as a control; w^{1118}, tj or tj>), *w; UAS EcRA.W650A TP3* (>EcRw^{DN}), *w; UAS EcR RNAi CA104* (>EcRi), *yw;+;UAS PTEN* (>PTEN), *yw flp; +; UAS InR29.4* (>InR), *w; UAS EcRA.W650A TP3; UAS InR29.4* (>EcRi, InR) or *w; UAS EcRA.W650A TP3; UAS InR29.4* (>EcR^{DN}, InR) . To characterize the expression patterns of *traffic jam*-GAL4, the following lines were used: *w; +; UAS GFP* (>GFP), *w; traffic jam*-GAL4; *UAS GFP* (tj>GFP), *w; elav-GAL4* (drives expression in neuroblast and glial cells in the larval brain; elav>) and *w; elav*-GAL80, *traffic jam*-GAL4 (inhibits *traffic jam*-GAL4 expression in the neuroblast and glial cells in the larval brain; elav80, tj>). Fly stocks were maintained at 22°C in bottles on standard fly food (4.5% molasses, 7.2% sugar, 7% cornmeal, 2% yeast extract, 1% agar and 2.5% Nipagin solution).

For ecdysone feeding experiments, I used an outbred population (wild type) of *D. melanogaster* established in the laboratory of Dr. Élio Sucena in 2007, originating from 160 fertilized females collected in Azeitão, Portugal (Martins et al., 2013). The population was kept in laboratory cages with high census (>1500 individuals) and maintained at constant temperature (25°C) on standard fly food (4.5% molasses, 7.2% sugar, 7% cornmeal, 2% yeast extract, 1% agar and 2.5% Nipagin solution).

3.2.2 Larval staging, dietary manipulations and ecdysone feeding experiments

Adults were allowed to lay eggs for two to six hours on fresh food plates (60 × 15 mm Petri dish). Egg density was controlled to prevent overcrowding (approximately 200 eggs per plate). To determine the effects of suppressing IIS and/or ecdysone signalling pathways in developing

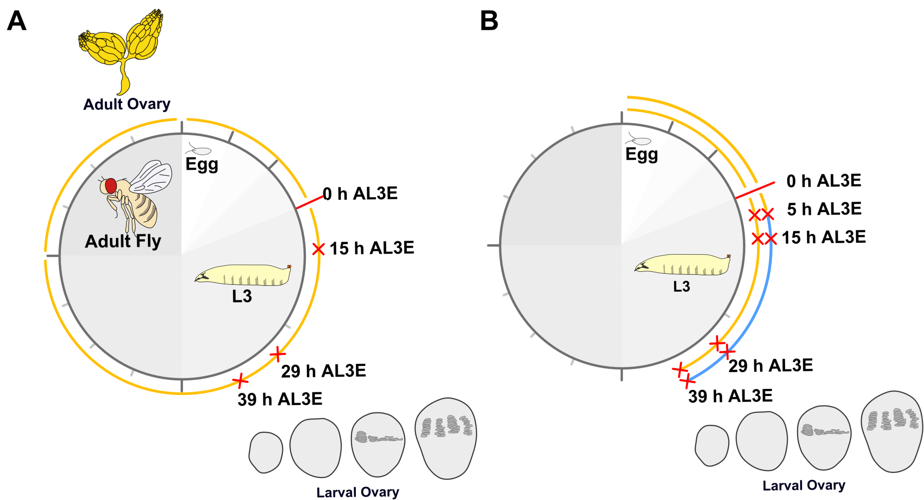


Figure 3.1: Experimental design of nutritional manipulations.(A) Experimental design to determine the effects of supressing IIS or ecdysone signalling on ovary development and ovariole number in larvae fed on standard food (yellow line). (B) A similar experimental design was performed to examine the effects of activating IIS and/or partially activating ecdysone signalling on ovary development. In this set of experiments, larvae were transferred at 5 h AL3E either to standard food (yellow line) or sucrose-only food (blue line). Dissection times are marked with red crosses.L3: third instar larvae; AL3E: after L3 ecdysis.

ovaries, larvae were selected 0-2 hours after ecdysis to L3 (AL3E) and transferred onto new plates (40-60 larvae per plate) with standard fly food (standard food) until the time of dissection (15 h, 29 h and 39 h AL3E) (Figure 3.1A). To determine the effects of activating IIS and/or partially activating ecdysone signalling pathways in developing ovaries, larvae were collected as above in 2 h intervals from ecdysis and fed on standard food. At 5 h AL3E, larvae were transferred to vials (20-30 larvae per vial) containing either 20% sucrose on 0.5% agar medium (sucrose-only food) or standard food. The sucrose-only food allowed most larvae to survive until pupariation and adult eclosion. Larval ovaries were dissected at four time points (5 h, 15 h, 29 h and 39 h AL3E) (Figure 3.1B).

For ecdysone feeding experiments, I supplemented 0.15 mg/mL of the active ecdysone metabolite 20-hydroxyecdysone (20E; SciTech Chemicals, Dejvice-Hanspaulka, Czech Republic) to 1 g of either standard food or sucrose-only food. The food was then well mixed and spun down a day before use. Newly ecdysed L3 larvae were collected as above and fed on

standard food for 5 hours. Larvae were then transferred to vials (20-30 larvae per vial) containing either 20E-supplemented standard food or 20E-supplemented sucrose-only food. As control, larvae at the same age were fed on either standard food or sucrose-only food supplemented with solvent ethanol. After 24 hours, larval ovaries were dissected. All experiments were performed at 25°C.

3.2.3 Measurements of life-history traits: developmental time, female weight and adult ovariole number

To determine the average time to pupariation, newly ecdysed L3 larvae were transferred to vials (20-30 larvae per vial) containing standard food. The number of larvae pupariating (immobile larvae with evaginated spiracles) was counted in 2 h intervals until all larvae pupariated. I used pharate weight as a proxy of adult body size (Mirth et al., 2005). Pharate adults were collected from food vials and food residuals were carefully cleaned off from pupal cases using distilled water and a paintbrush. I distinguished between females and males by the presence or absence of male-specific sex combs through the pupal case. Female pharate adults were individually weighed on a Sartorius SE2 ultramicrobalance. To count adult ovariole number, virgin flies were maintained in vials (ten females and five males per vial) with standard food until the time of dissection (4-6 days after eclosion). Ovaries were dissected in cold phosphate buffered saline containing 1% Triton X-100 (PBT) and ovarioles were teased apart and counted under a stereoscope.

3.2.4 Immunocytochemistry, imaging and analysis

Immunocytochemistry was performed as previously described (see Chapter 2). I used the primary antibody mouse anti-Engrailed (Developmental Studies Hybridoma Bank 4D9, 1:40) to identify terminal filament cells (TFCs). The following secondary reagents were used: Alexa 568 (Invitrogen, 1:200) and TRICT-Phalloidin (Sigma, 1:200). Samples were mounted on a poly-L-lysine-coated coverslip using Fluoromount-G (SouthernBiotech) and imaged using a Zeiss LSM 510 Meta confocal

microscope. Measurements of total number of forming terminal filaments (TFs) and ovary volume were performed as previously described in Chapter 2 using ImageJ (NIH) and Adobe Photoshop (Adobe Systems).

3.2.5 Statistical Analysis

All experiments were replicated at least twice. The distribution of residuals was tested for normality using Q-Q plots and the appropriate statistical test was applied. Welch two sample t-test or Wilcoxon rank sum test were used to test differences in mean values between two samples for normally and non-normally distributed residuals, respectively. Holm's *p*-value adjustment was performed when necessary. Differences in the rate of TF formation and of ovary growth were tested using ANCOVAs or the function 'sm.ancova' under 'sm' library when distribution of residuals was non-normal. Differences in the timing of the onset of TFC differentiation were tested with a Chi-squared test. All data analyses and statistics were conducted using R v3.1.2 (R Development Core Team, 2014). Plots were made using GraphPad Prism v6 (GraphPad Software). Statistical tests and *p*-values are indicated in the text and figures.

3.3 Results

3.3.1 Ovariole number is regulated by IIS and ecdysone signalling

Supressing IIS or ecdysone signalling pathway in the whole organism or specifically in ovarian somatic cells reduces TF number and ovary size (Gancz and Gilboa, 2013; Green and Extavour, 2012, 2014). As changes in the dynamics of TF formation and ovary growth influence adult ovariole number (see Chapter 2) (Green and Extavour, 2014; Hodin and Riddiford, 2000; Sarikaya and Extavour, 2015; Sarikaya et al., 2012), I asked whether manipulating IIS or ecdysone signalling pathways in the larval ovary affects adult ovariole number.

Before genetically manipulating the IIS and/or ecdysone signalling pathways in the larval ovary, I first characterized the expression profiles of

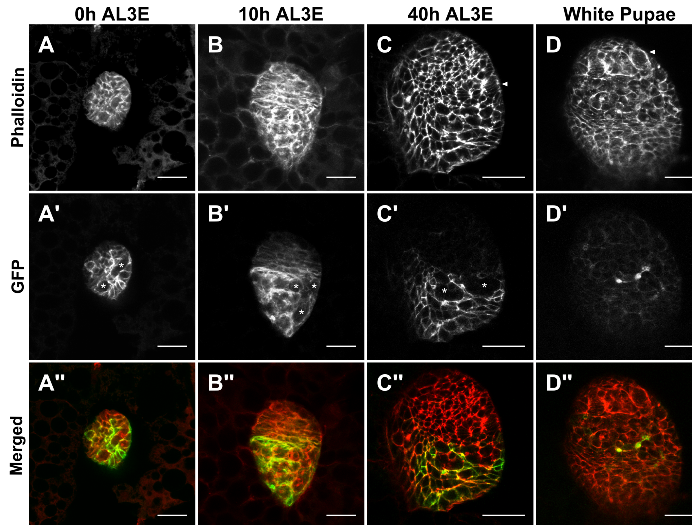


Figure 3.2: *traffic jam*-GAL4 is expressed in ovarian somatic cells during L3 larval stages. (A, B, C, D and A", B", C", D") Phalloidin marks F-actin to outline cell membranes (grey, red). (A', B', C', D' and A", B", C", D") GFP reporter line under the control of *traffic jam*-GAL4 driver line (grey, green). Scale bar: 20µm. L3: third instar larvae; AL3E: after L3 ecdysis. In (C, D), white arrowheads denote forming terminal filaments. In (A', B' and D'), asterisks denote germ cells.

the *traffic jam*-GAL4 driver. At 0 h AL3E, *traffic jam*-GAL4 is expressed in all somatic cells of the larval ovary, but not in germ cells. Throughout L3 larval development, its expression becomes gradually restricted to the posterior part of the ovary. At pupariation, only few cells in the posterior part of the ovary express *traffic jam*-GAL4 (Figure 3.2). Furthermore, I found that *traffic jam*-GAL4 is also expressed in the larval brain (Figure 3.3A-B"). To determine which cells in the larval brain express *traffic jam*-GAL4, I used *elav*-GAL80 to inhibit expression of *traffic jam*-GAL4 in the neurons and neuroblasts in the larval brain (elav80, tj>GFP). This abolished all expression in the larval brain, but not in the larval ovary, indicating that *traffic jam*-GAL4 expression in the larval brain is solely in the neurons and neuroblasts (Figure 3.3C-D").

To determine whether suppressing IIS in the larval ovary reduces ovariole number, I used *traffic jam*-GAL4 driver line to overexpress Phosphatase and tensin homolog (PTEN) under the control of UAS (tj>PTEN). Adult ovariole number in tj>PTEN adult flies was significantly reduced

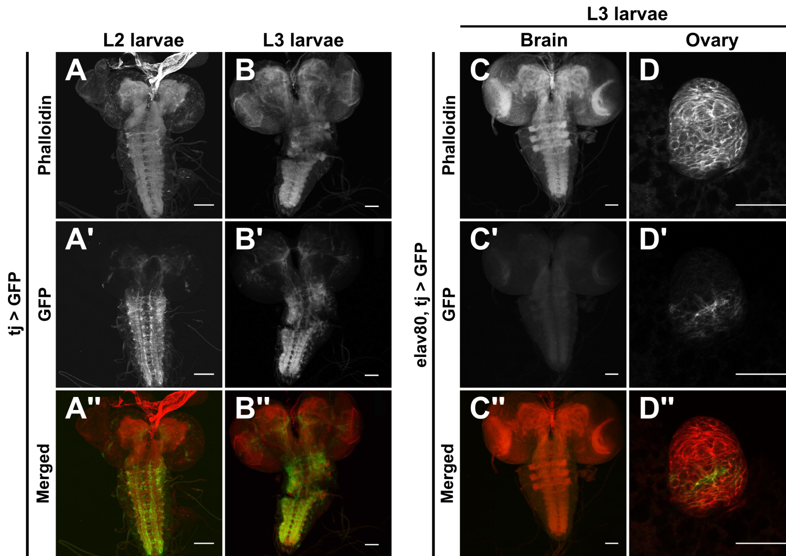


Figure 3.3: *traffic jam*-GAL4 is expressed in neuroblasts and glial cells in the larval brain. (A, B, C, D and A", B", C", D") Phalloidin marks F-actin to outline cell membranes (grey, red). (A', B' and A", B") GFP reporter line under the control of *traffic jam*-GAL4 driver line (grey, green). Scale bar: 50µm. L2: second instar larvar; L3: third instar larvae.

(Figure 3.4A). Also, tj>PTEN larvae developed faster and gave rise to smaller adults when compared to controls (Figure 3.4B, C). As *traffic jam*-GAL4 is expressed in the larval brain, I next asked whether the effects in ovariole number in tj>PTEN adult females were due to a reduction in IIS activity in the larval brain. To test this prediction, I overexpressed PTEN specifically in ovarian somatic cells (elav80, tj>PTEN) or in neuroblasts and neurons of the larval brain (elav>PTEN). As expected, ovariole number was significantly reduced in elav80, tj>PTEN females (Figure 3.4D). On the other hand, elav>PTEN females had a similar number of ovarioles as control females (Figure 3.4D). These results indicate that suppressing IIS pathway in the neuroblasts and neurons of the larval brain has no effect on ovariole number. Nonetheless, the reduction in ovariole number was stronger in tj>PTEN females than in elav80, tj>PTEN females ($p<0.001$; Welch two sample t-test). These differences are likely caused by genetic background. Interestingly, pharate weight was reduced in both tj>PTEN and elav80, tj>PTEN females (Figure 3.4E).

This reduction in body size was not observed in elav>PTEN females (Figure 3.4E).

Ecdysone binds to EcR/Usp to induce two types of functions (Cherbas, 2003). First, for genes that are repressed by unliganded EcR/Usp, ecdysone relieves this repression (i.e. derepression) and allows gene transcription (Brown et al., 2006; Schubiger and Truman, 2000; Schubiger et al., 2005). Secondly, by binding to EcR/Usp, ecdysone activates the transcription of target genes (i.e. activation) (Cherbas, 2003; Hu et al., 2003). To determine the effects of supressing ecdysone signalling on ovariole number, I overexpressed a dominant negative form of EcR (EcR^{DN}) under the control of UAS (tj>EcR^{DN}). This EcR^{DN} is mutated in the ligand-binding domain and thus its overexpression abolishes both the derepression and activation functions of ecdysone (Brown et al., 2006; Cherbas, 2003; Hu et al., 2003). Most tj>EcR^{DN} larvae died in pupal stages. The few tj>EcR^{DN} females that eclosed had defects in ovariole structure and were sterile. However, I was able to identify individual ovarioles and count them in these females. Ovariole number was severely reduced in tj>EcR^{DN} adult females (Figure 3.4A). Moreover, tj>EcR^{DN} larvae accelerated the onset of metamorphosis and gave rise to smaller adults when compared to controls (Figure 3.4B, C).

3.3.2 Role of IIS during ovary development

Two recent studies have shown that IIS determines TF number by regulating ovary growth (Gancz and Gilboa, 2013; Green and Extavour, 2012). However, the proliferation rates of somatic cells are not constant during ovary development (Sahut-Barnola et al., 1996) and thus, a detailed description of how IIS affects ovary volume through L3 larval stages is needed to fully understand the role of IIS in regulating ovariole number. Moreover, IIS controls the timing of crucial differentiation events in the eye and leg imaginal discs (Bateman and McNeill, 2004; McNeill et al., 2008). Therefore, I asked whether IIS regulates the three nutrition-sensitive processes during ovary development: the onset of TFC differentiation, the rate of TF formation, and the rate of ovary growth.

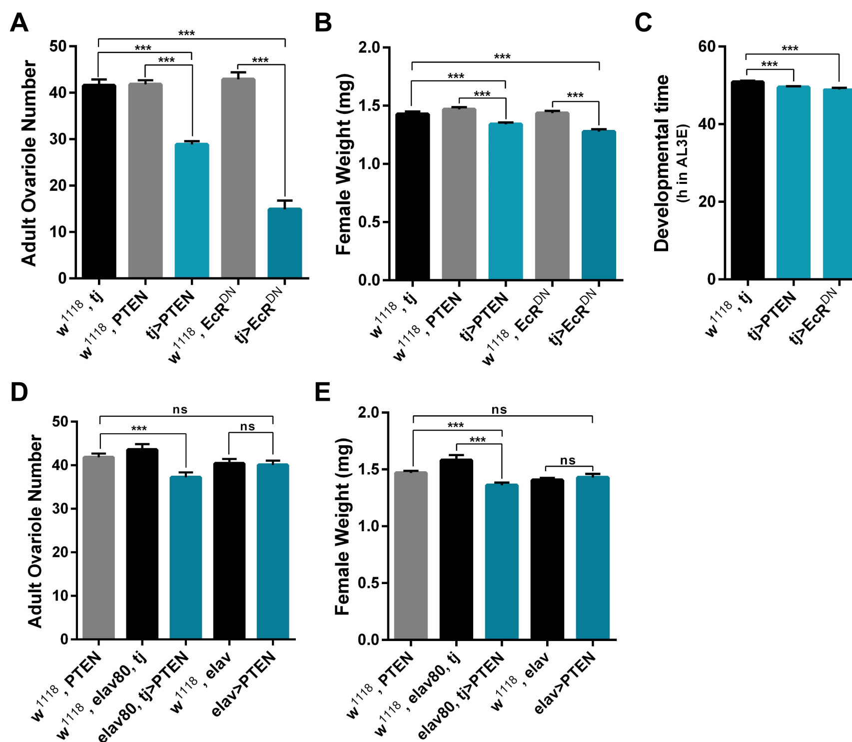


Figure 3.4: Manipulating IIS or ecdysone signalling in the larval ovary reduces adult ovariole number and female weight. (A) Adult ovariole number, (B) female pharate weight and (C) developmental times represented in hours after third instar ecdysis (h AL3E) to pupariation of individuals with disruption of IIS or ecdysone signalling under the control of *traffic jam*-GAL4 driver line (tj>PTEN and tj>EcR^{DN}, respectively; blue bars). (D) Adult ovariole number and (B) pharate weight of females with disruption of IIS in ovarian somatic cells (elav80, tj>PTEN; blue bar) or in neuroblasts and glial cells (elav>PTEN; blue bar). Controls are either driver (black bars) or reporter (grey bar) lines crossed with wild-type line (w^{1118}). n $\geq$ 20 females for all genotypes. Plotted values represent means and error bars show 95% confidence intervals of means. Welch two sample t-test using Holm's *p*-value adjustment: ***p<0.001, ns non-significant.

I first analysed ovary development in tj>PTEN larvae reared in standard food conditions. Ovaries from tj>PTEN larvae showed a moderate delay in the onset of TFC differentiation (Figure 3.5A, B). Further, both rate of TF formation and the rate of ovary growth were greatly reduced in tj>PTEN ovaries when compared to control ovaries (w1118; tj) (Figure-3.5A-A”, B-B”, D, E). Conversely, activating IIS in ovarian somatic cells by overexpressing Insulin Receptor (tj>InR) did not affect the timing of TFC differentiation (Figure-3.5A, C), although it increased the rate of TF formation and of ovary growth (Figure 3.5A-A”, C-C”, D, E). Overall, these results suggest that IIS regulates all three developmental processes in well-fed conditions.

I next sought to test whether activation of IIS was sufficient to overcome the effects of poor nutrition in larvae fed on sucrose alone. In control larvae fed on sucrose, I failed to detect any TFCs before 39 h AL3E (Figure 3.5F’, F”). In contrast, I detected TFCs in tj>InR ovaries from larvae fed on sucrose already at 15 h AL3E (Figure 3.5G’). Such timing of TFC onset in sucrose fed tj>InR larvae was similar to that of well-fed controls (Figure 3.5A, C, G’, H) and new TFs were still forming at 39 h AL3E (Figure 3.5G”, H).

The rate of TF formation was significantly higher in tj>InR larvae fed on sucrose alone than in similarly treated control larvae. Further, at the time of transfer to sucrose-only food (5 h AL3E), the ovary volume in tj>InR larvae was significantly bigger than that of ovaries from control larvae ($p<0.001$; Kruskal-Wallis test). However, when tj>InR larvae were fed on sucrose-only food between 5 and 39 h AL3E, ovary growth was completely arrested (Figure 3.5I). In summary, activation of IIS in ovarian somatic cells of poorly-fed larvae is sufficient to induce a precocious onset of TFC differentiation and to accelerate the rate of TF formation, but has no effect on ovary growth.

3.3.3 Role of ecdysone signalling during ovary development

My previous manipulations of larval nutrition revealed that the onset of TFC differentiation is highly sensitive to changes in nutrition during the

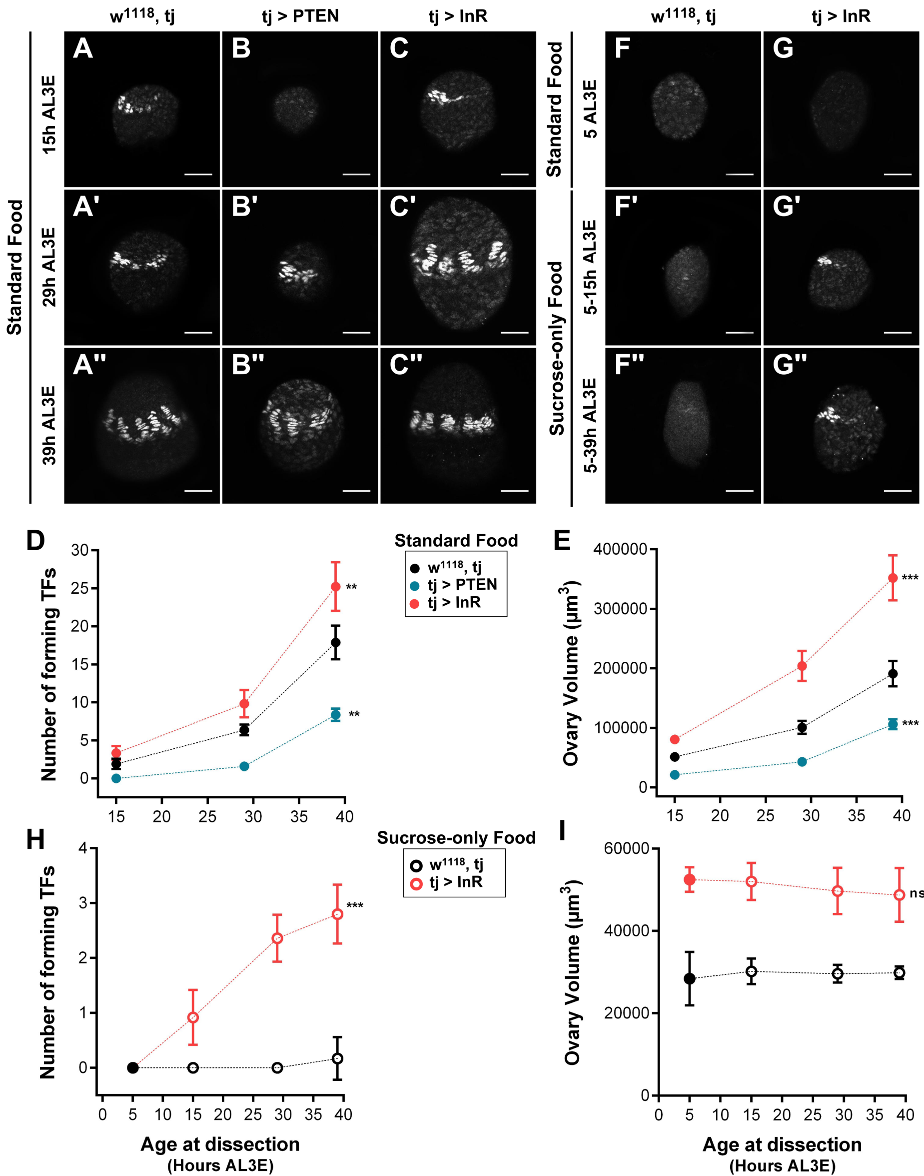


Figure 3.5: Role of IIS during ovary development. (A-G") Shown is terminal filaments (TFs) marked with En (grey).(A-C", F, G) Ovaries from larvae reared on standard food: (A-A", F) w1118, tj (control), (B-B") tj>PTEN and (C-C", G) tj>InR.(D) Number of forming TFs and (E) ovary volume of ovaries from larvae reared on standard food. (F'-F", G'-G") Ovaries from larvae transferred to sucrose-only food at 5 h AL3E: (F'-F") w1118, tj (control) and tj>InR. (F'-F").(H) Number of forming TFs and (I) ovary volume of ovaries from larvae fed on sucrose-only food. n ≥ 8 ovaries for all genotypes. Plotted values represent means and error bars show 95% confidence intervals of means. In some cases, error bars are too small to be seen. ANCOVAs: **p<0.01, ***p<0.001, ns non-significant. L3: third instar larvae; AL3E: after L3 ecdysis. Scale bar: 20µm.

pre-critical weight phase. Once TFC differentiation is initiated around the timing of critical weight, TF formation proceeds at a reduced rate in poorly-fed larvae (see Chapter 2). Critical weight itself is regulated by a small nutrition-sensitive ecdysone peak that occurs at around 8 h AL3E (Koyama et al., 2014; Mirth et al., 2005; Warren et al., 2006). Moreover, both EcR and Usp are expressed in ovarian somatic cells during L3 larval stages (Gancz et al., 2011; Hodin and Riddiford, 1998). Thus, I reasoned that ecdysone is likely to induce TFC differentiation.

Under standard food conditions, control ovaries (w^{1118}, tj) showed TFCs and a few forming TFs at 15 h AL3E (Figure 3.6A-A", D). However, I did not detect any TFCs in tj>EcR^{DN} ovaries from well-fed larvae until 39 h AL3E (Figure 3.6B-B", D). Accordingly, the rate of TF formation was severely reduced in tj>EcR^{DN} ovaries (Figure 3.6D). Thus, ecdysone signalling is necessary to induce the timely onset of TFC differentiation and promote subsequent formation of new TFs. In addition, I found that the rate of ovary growth was significantly reduced in tj>EcR^{DN} ovaries (Figure 3.6E), suggesting that basal levels of ecdysone are likely required to promote ovary growth.

I then tested whether a partial activation of ecdysone signalling in ovarian somatic cells is sufficient to induce the onset of TFC differentiation, to increase the rate of TF formation, and promote ovary growth in pre-critical weight larvae fed on sucrose-only food. To do this, I used the *traffic jam*-GAL4 driver line to overexpress an RNAi construct against EcR under the control of UAS (tj>EcRi). In well-fed conditions, tj>EcRi ovaries showed similar timing in their onset of TFC differentiation as well as rate of TF formation relative to controls (Figure 3.6A, C, D). The rate of ovary growth was reduced in tj>EcRi ovaries (Figure 3.6E). When tj>EcRi larvae were fed on sucrose-only food between 5 and 15 h AL3E, most ovaries had TFCs (Figure 3.6G', H). In contrast, TFCs were not detectable in control (w^{1118}, tj) larvae fed on sucrose-only food until 39 h AL3E (Figure 3.6F', H). Also, the rate of TF formation, but not of ovary growth, increased in tj>EcRi larvae fed on sucrose alone (Figure 3.6H, I). Together, these results indicate that knocking down EcR in the ovarian somatic cells of larvae fed on sucrose alone is sufficient to induce precocious

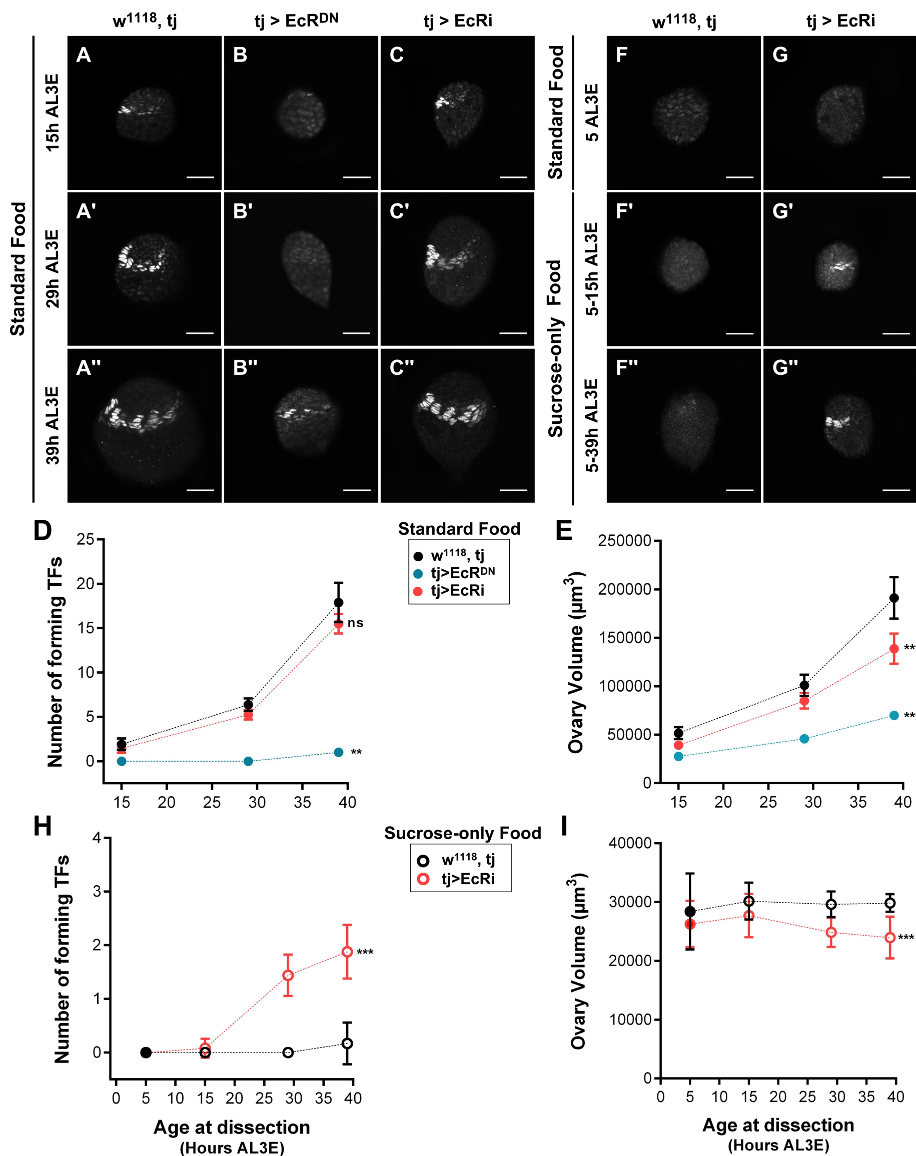


Figure 3.6: Role of ecdysone signalling during ovary development. (A-G”) Shown is terminal filaments (TFs) marked with En (grey).(A-C”, F, G) Ovaries from larvae reared on standard food: (A-A”, F) w^{1118}, tj (control), (B-B”) tj>EcR^{DN} and (C-C”, G) tj>EcRi.(D) Number of forming TFs and (E) ovary volume of ovaries from larvae reared on standard food. (F’-F”, G’-G”) Ovaries from larvae transferred to sucrose-only food at 5 h AL3E: (F’-F”) w^{1118}, tj (control) and (G’-G”) tj>EcRi. (H) Number of forming TFs and (I) ovary volume of ovaries from larvae fed on sucrose-only food. $n \geq 8$ ovaries for all genotypes. Plotted values represent means and error bars show 95% confidence intervals of means. In some cases, error bars are too small to be seen. ANCOVAs: **$p<0.01$, ***$p<0.001$, ns non-significant. L3: third instar larvae; AL3E: after L3 ecdysis. Scale bar: 20µm.

onset of TFC differentiation and accelerate the rate of TF formation when compared to similarly treated controls. However, knocking down EcR in either standard or sucrose-only conditions reduces ovary growth.

Knocking down EcR in the larval ovaries induces the derepression, but not the activation function of ecdysone signalling (Brown et al., 2006; Schubiger and Truman, 2000; Schubiger et al., 2005). To investigate the full role of ecdysone signalling in regulating ovariole number plasticity, I fed wild-type larvae from 5 to 29 hours AL3E on either standard food or sucrose-only food supplemented with 0.15 mg/mL of the active ecdysone metabolite 20-hydroxyecdysone (20E). Adding 20E to the standard food had no effect on TF number (Figure 3.7A, C, E). However, larvae fed on 20E-supplemented sucrose-only food had significantly more TFs at 29 hours AL3E than larvae fed on sucrose-only food plus solvent (ethanol) (Figure 3.7B, D, E). Moreover, ovary volume significantly increased in larvae fed on both standard and sucrose-only foods containing 20E relative to ethanol controls (Figure 3.7F). This experiment confirms that ecdysone is sufficient to induce TFC differentiation when pre-critical weight larvae are fed on sucrose alone. Moreover, it also suggests that ecdysone regulates ovary growth through its activation function.

3.3.4 The interplay between IIS and ecdysone signalling pathways

Both IIS and ecdysone signalling pathways regulate the onset of TFC differentiation, the rate of TF formation, and the rate of ovary growth, suggesting that nutrition influences ovariole number through both signalling pathways. However, the relative contribution of these signalling pathways in regulating each development process appears to be different, with ecdysone signalling playing a more prominent role in regulating TFC differentiation and IIS contributing more to the rates of ovary growth.

To test this hypothesis, I first upregulated IIS, using InR, while inhibiting ecdysone signalling, via EcR^{DN}, in ovarian somatic cells (tj>EcR^{DN}, InR) of well-fed larvae. Ovaries from tj>EcR^{DN}, InR larvae delayed the onset of TFC differentiation and reduced the rate of TF formation when compared

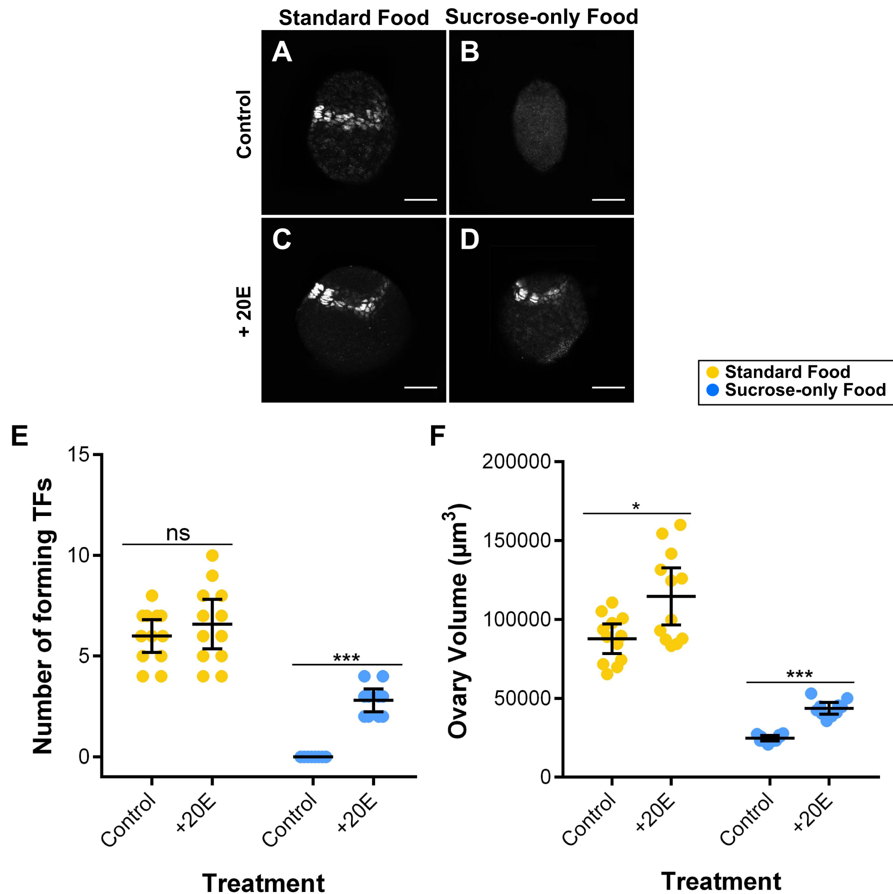


Figure 3.7: Feeding wild-type larvae with 20E-supplemented sucrose-only food increases TF number and ovary volume. (A-D) Shown is terminal filaments (TFs) marked with En (grey). Ovaries from larvae reared on standard food: (A) plus ethanol (control) or (C) plus 20E (+20E). Ovaries from larvae reared on sucrose-only food: (B) plus ethanol (control) or (D) plus 20E (+20E). Larvae were dissected at 29 h AL3E. Scale bar: 20μm. (E) Number of forming TFs and (F) ovary volume of ovaries from larvae fed either on standard food plus ethanol (control) or on 20E-supplemented standard food (+20E) (yellow points) and larvae fed either on sucrose alone plus ethanol (control) or on 20E-supplemented sucrose-only food (+20E) (blue points). Plotted values represent means and error bars show 95% confidence intervals of means. In some cases, error bars are too small to be seen. Welch Two sample t-test: *$p<0.1$, ***$p<0.001$, ns non-significant.

to control larvae (w^{1118}; tj) (Figure 3.8A-A", 3.8B-B", D). Thus, to induce TFC differentiation, IIS requires ecdysone signalling to be intact. Nevertheless, the rate of ovary growth increased relative to control and tj> EcR^{DN} larvae (Figure 3.8E and Table 3.1). Taken together, these results demonstrate that ecdysone signalling is essential in regulating the timing of the onset of TFC differentiation. Furthermore, these results also show that IIS can overcome the growth defects arising from disrupting ecdysone signalling, suggesting that IIS plays a primary role in controlling ovary growth.

I next tested the effects of upregulating both signalling pathways on each developmental process. I partially activated ecdysone signalling, using EcRi, while upregulating IIS, with InR, in ovarian somatic cells (tj>EcRi, InR). Interestingly, TFCs were observed at 5 h AL3E in ovaries from tj>EcRi, InR fed on standard food (Figure 3.8G). This onset of TFC differentiation was not only earlier than that of control larvae (w^{1118}; tj) (Figure 3.8), it was also significantly earlier than the onset of TF formation in tj>InR and tj>EcRi ovaries ($p<0.0001$, $\chi^2 = 45$, df = 3, Chi-Square Test) (Figure 3.5G and Figure 3.6G). The rate of TF formation and of ovary growth was faster in ovaries from tj>EcRi, InR larvae than ovaries from either control or tj>EcRi larvae (Figure 3.8D, E and Table 3.2). Remarkably, even though ovaries grew at the same rate in both tj>EcRi, InR and tj>InR larvae, the rate of TF formation was faster in tj>EcRi, InR ovaries (Table 3.2).

In summary, activating both IIS and ecdysone signalling pathways in ovarian somatic cells of well-fed larvae induced an earlier onset of TFC differentiation, and promoted a greater increase in the rate of TF formation than activating each signalling pathway individually (Table 3.1 and 3.2). This led me to hypothesize that activating both signalling pathways may overcome most of the effects of poor nutrition. When tj>EcRi, InR larvae were fed on sucrose-only food between 5 and 15 hours AL3E, the rate of TF formation was significantly faster than the control (w^{1118}; tj) ovaries (Figure 3.8H). In fact, the rate of TF formation in ovaries from tj>EcRi, InR larvae was faster than all previous genetic manipulations in poorly fed larvae (Table 3.3). While at 5 hours AL3E

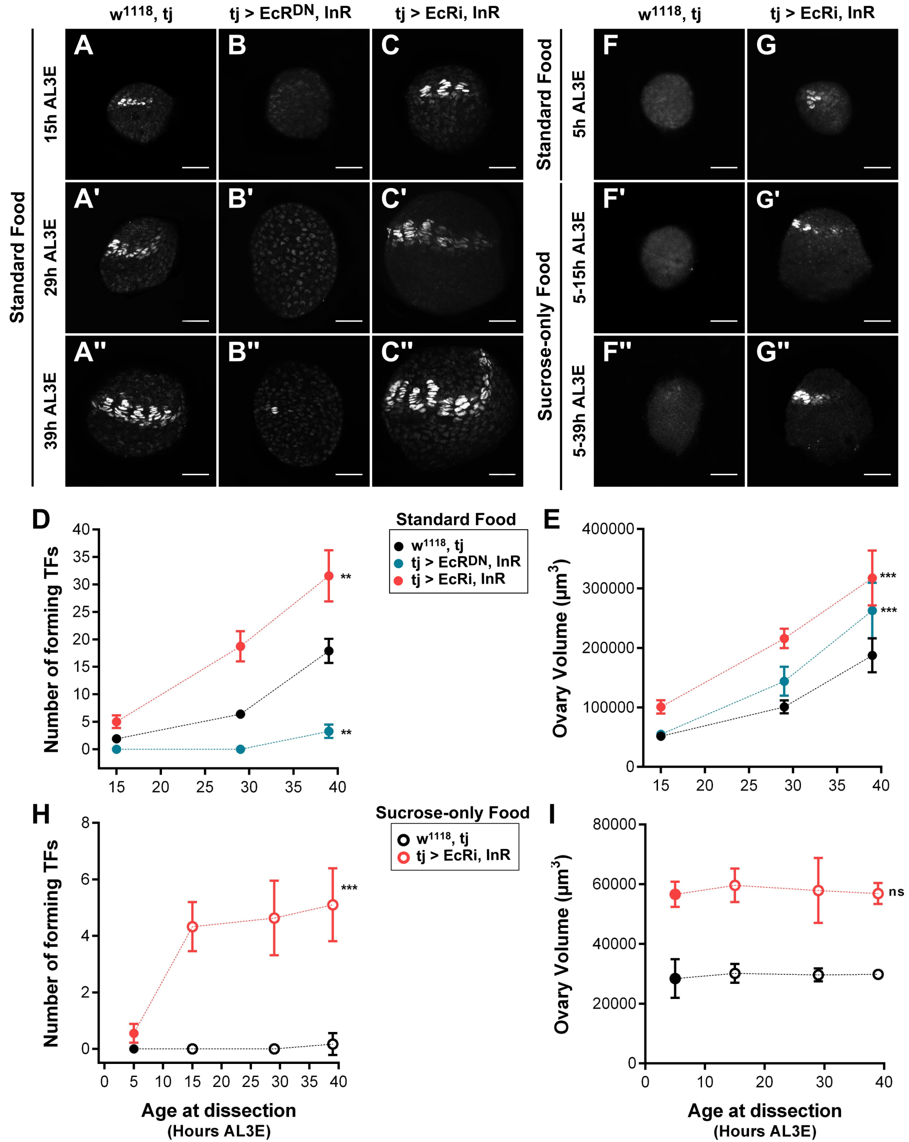


Figure 3.8: The interplay between IIS and ecdysone signalling pathways. (A-G”) Shown is terminal filaments (TFs) marked with En (grey).(A-C”, F, G) Ovaries from larvae reared on standard food: (A-A”, F) w^{1118}, tj (control), (B-B”) tj>EcR^{DN}, InR and (C-C”, G) tj>EcRi, InR.(D) Number of forming TFs and (E) ovary volume of ovaries from larvae reared on standard food. (F’-F”, G’-G”) Ovaries from larvae transferred to sucrose-only food at 5 h AL3E: (F’-F”) w^{1118}, tj (control) and (G’-G”) tj>EcRi, InR. (H) Number of forming TFs and (I) ovary volume of ovaries from larvae fed on sucrose-only food. $n \geq 8$ ovaries for all genotypes. Plotted values represent means and error bars show 95% confidence intervals of means. In some cases, error bars are too small to be seen. ANCOVAs: $^{**}p<0.01$, $^{***}p<0.001$, ns non-significant. L3: third instar larvae; AL3E: after L3 ecdysis. Scale bar: 20µm.

the ovary volume of tj>EcRi, InR ovaries was bigger than the control (p<0.001, Kruskal-Wallis test), I did not observe any further increase in ovary volume after transferring tj>EcRi, InR larvae to sucrose-only food (Figure 3.8I). Together, these results show that, in poor nutritional conditions, activating both IIS and ecdysone signalling pathways in ovarian somatic cells increases the rate of TF formation, but not ovary growth.

Table 3.1: Pairwise comparisons of the rate of ovary growth in larvae fed on standard food. Shown are *p*-values (** p<0.01, ***p<0.001, ns non-significant; ANCOVAs using Holm's *p*-value adjustment) for each pairwise comparison.

Genotypes	tj>EcR^{DN}, InR	tj>EcRi, InR
w^{1118},tj	0.0001 ***	0.0001 ***
tj>EcR^{DN}	0.0014 **	-
tj>EcRi	-	0.0007 ***
tj>InR	0.0014 **	0.8450 ns
tj>EcRi, InR	0.0005 ***	-

Table 3.2: Pairwise comparisons of the rate of TF formation in larvae fed on standard food. Shown are *p*-values (** p<0.01, ***p<0.001, ns non-significant; ANCOVAs using Holm's *p*-value adjustment) for each pairwise comparison.

Genotypes	tj>EcR^{DN}, InR	tj>EcRi, InR
w^{1118},tj	0.0026 **	0.0029 **
tj>EcR^{DN}	0.0055 **	-
tj>EcRi	-	0.0055 **
tj>InR	0.0030 **	0.0055 **
tj>EcRi, InR	0.0029 **	-

Table 3.3: Pairwise comparisons of the rate of TF formation in larvae fed on sucrose alone. Shown are *p*-values (** p<0.01, ***p<0.001, ANCOVAs using Holm's *p*-value adjustment) for each pairwise comparison.

Genotypes	tj>EcRi, InR
w^{1118},tj	0.0001 ***
tj>EcRi	0.0017 **
tj>InR	0.0017 **

3.4 Discussion

Hormonal regulation underlies most, if not all, well-studies cases of developmental plasticity (Beldade et al., 2011). However, how hormones change an organ's response to environmental conditions over developmental time and how this influences their plastic response are poorly understood. In this chapter, I provide evidence that two hormonal signalling pathways – the IIS and ecdysone signalling pathways – act during critical weight to change the sensitivity of the developing ovary to nutritional conditions and thus, regulate the plastic response of ovariole number.

In the previous chapter, I have shown that pre-critical weight larvae fed on sucrose-only food delay the onset of TFC differentiation (see Chapter 2). Both IIS and ecdysone signalling pathways regulate the onset of TFC differentiation; altering either IIS or ecdysone signalling in the ovary altered the timing of the onset of TFC differentiation. However, the effects of IIS on the onset of TFC differentiation depended on ecdysone signalling. Ovaries in which IIS was upregulated while ecdysone signalling was repressed delayed the onset of TFC differentiation as much as ovaries in which only ecdysone signalling was repressed.

Even though ecdysone signalling was required to induce the onset of TFC differentiation, the two pathways appear to interact in a complex manner to regulate this process. Simultaneously upregulating both IIS and ecdysone signalling in the ovary resulted in earlier onset of TFC differentiation than upregulating either pathway on its own. Potentially, nutrition, via IIS, may modify the sensitivity of the ovary to ecdysone signalling. Under high levels of IIS, the ovary may require lower levels of ecdysone signalling to induce the onset of TFC differentiation, resulting in earlier onset. Further studies will be required to fully understand the nature of the link between IIS and ecdysone signalling in this developmental process.

Additionally, my results suggest that unliganded EcR/Usp represses genes involved in the onset of TFC differentiation. Knocking down EcR in the ovary eliminates the repressive function of the receptor, and induces

premature onset of TFC differentiation in pre-critical weight larvae fed on sucrose alone. Similarly, patterning in the wing disc of pre-critical weight larvae is repressed by unliganded EcR/Usp (Mirth et al., 2009). Therefore, the critical weight ecdysone peak relieves the repressive effects of unliganded EcR/Usp, causing a derepression of the genes involved in inducing the onset TFC differentiation in the ovary and of those necessary for the continued patterning of the wing (Mirth et al., 2009).

Both IIS and ecdysone signalling appear to be involved in the rate of TF formation as repressing either IIS or ecdysone signalling reduced the rates of TF formation. However, the effects of ecdysone signalling on the rate of TF formation may be partly due to delays in the onset of TFC differentiation. While activating IIS increased the rate of TF formation in standard and sucrose-only foods, activating ecdysone signalling only increased the rate of TF formation under two conditions: i) in larvae fed on sucrose-only food, presumably due to precocious TFC differentiation, and ii) when both IIS and ecdysone signalling where activated in the larval ovary. These results suggest that increased ecdysone signalling can accelerate the rate of TF formation when IIS is high.

Manipulating IIS alters the rate of ovary growth in optimal nutritional conditions. Despite this, activation of IIS failed to promote ovary growth in larvae fed on sucrose alone. A second nutrient-sensitive pathway, the target of rapamycin (TOR) pathway, responds directly to intracellular concentrations of amino acids to promote growth (Gao et al., 2002). Inactivating components of the TOR signalling pathway leads to a reduction in ovary volume (Gancz and Gilboa, 2013) and thus, its activation might be sufficient to induce ovary growth on sucrose-only food. Alternatively, and most likely, ovary growth may depend directly on the availability of amino acids. Therefore, under protein-starved conditions, ovary growth is halted even when IIS is upregulated.

By genetically manipulating ecdysone signalling, the rate of ovary growth was reduced and, similar to IIS, partially activating ecdysone signalling was not sufficient to promote ovary growth in poorly fed larvae. However, feeding larvae with 20E slightly increased ovary volume in both standard and sucrose-only food conditions. In the lepidopteran, *Manduca sexta*,

a decline in circulating levels of the juvenile hormone at critical weight and the subsequent increase in ecdysone titeres initiate a morphogenetic growth of the imaginal discs (Truman et al., 2006). Such growth occurs independently of nutritional inputs (Tobler and Nijhout, 2010). Thus, rising levels of ecdysone in larvae fed on 20E-supplemented food might have promoted nutrition-insensitive growth in the larval ovary. This increase in ovary volume in larvae fed in 20E-supplemented food may also be due to systemic effects in the whole larvae. In either case, ecdysone likely regulates growth in the developing ovary through the activation function.

3.5 Conclusions

By using nutritional, hormonal, and genetic manipulations, I found that IIS and ecdysone signalling pathways change the developing ovary's sensitivity to larval nutrition by controlling three stage-specific developmental processes: the onset of TFC differentiation, the rate of TF formation, and the rate of ovary growth. However, the relative contribution of IIS and ecdysone signalling pathways in regulating each developmental process in response to nutrition was different. At critical weight, ecdysone plays a major role in controlling the timing of the onset of TFC differentiation. However, IIS potentially tunes the timing of the onset of TFC differentiation by modulating the response of the developing ovary to ecdysone signalling. Conversely, both rate of TF formation and of ovary growth are predominantly regulated by IIS with ecdysone signalling playing a secondary role.

Acknowledgments

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4

EVOLUTIONARY DIVERGENCE IN OVARIOLE NUMBER IN *DROSOPHILA MOJAVENSIS* SUBSPECIES

"If artificial selection can work such profound changes in only ten or fifteen thousand years, what can natural selection do operating over billions of years? The answer is all the beauty and diversity of life."

– from the documentary TV series *Cosmos:A Spacetime Odyssey* (2014)

Abstract

Several authors have proposed that developmental plasticity facilitates evolutionary change by providing a range of novel and distinct phenotypes in response to environmental variation. Comparing the developmental basis of both plastic responses and inter/intraspecific genetic variation for a trait would serve to elucidate how developmental plasticity contributes to evolutionary processes. The second and third chapters of this thesis investigated how nutrition modifies three developmental processes during ovary development – the onset of terminal filament cell (TFC) differentiation, the rate of terminal filament (TF) formation, and of ovary growth – to generate diversity in ovariole number. In this chapter, I explored whether similar developmental processes can account for differences in ovariole number between two subspecies of *D. mojavensis*, *D. moj. sonorensis* and *D. moj. wrigleyi.* This characterization revealed that all three developmental processes differ between subspecies. Nevertheless, divergence in ovary growth rates appears to underlie much of the variation in ovariole number. Interestingly, ovariole number showed a similar response to nutritional variation in both *D. moj. sonorensis* and *D. moj. wrigleyi*, suggesting that divergence in ovariole number is due to genetic variation in the number of ovarioles and not to differences in nutritional plasticity. I discussed these findings in the context of my previous results on nutritional plasticity and the present literature on ovariole number plasticity and evolution.

Publication

A version of this chapter is part of a manuscript in preparation, authored by C.C.Mendes, E. Sucena and C.K.Mirth.

Authors' contributions

Christen Mirth, Élio Sucena and Cláudia Mendes conceived this study. Cláudia Mendes performed and analysed all experiments and wrote this chapter.

4.1 Introduction

In recent years, it has become increasingly clear that the environment not only selects among phenotypes, but it also alters developmental trajectories, sometimes in profound ways, to generate astonishing variation in morphology, behaviour, physiology and life history (Beldade et al., 2011; Pfennig et al., 2010; Stearns, 1989). Such environmentally-induced traits may allow the persistence of a population in a novel and challenging environment, and can gradually become stabilized and integrated with the rest of the phenotype by means of genetic accommodation (West-Eberhard, 2003). A possible outcome from such adaptive refinement of environmentally-induced phenotypes is that the developmental processes that generate phenotypic variation among populations should be to some degree similar to the developmental changes that emerge as a response to environmental variation (Wund, 2012).

The remarkable plastic capacity, paired with a substantial genetic variation both within and between species, makes ovariole number in *Drosophila* an exciting model to address this issue. In previous chapters, I examined how larval nutrition shapes ovariole number in an outbred population of *D. melanogaster*. A detailed characterization of ovary development during third instar (L3) larval stages revealed that nutrition influences ovariole number by altering three distinct developmental processes during ovary development: i) the onset of terminal filament cell (TFC) differentiation, which marks the beginning of ovariole development, ii) the rate at which new terminal filaments (TF) are formed, and iii) the rate of ovary growth. Importantly, the nutrition-dependent developmental transition, critical weight, alters the types of developmental processes nutrition can affect: while starving larvae before critical weight delays the onset of TFC differentiation and ovary growth, changes in nutrition after critical weight reduce the rates of ovary growth and of TF formation. Here, I sought to explore whether changes in one or all of the three developmental processes are responsible for the divergence in ovariole number among *Drosophila* species.

The intra/interspecific variation in ovariole number is correlated with the colonization of diverse ecological niches. In general, *Drosophila* species

that feed on a narrow range of food sources have fewer ovarioles than species with a generalist diet (Green and Extavour, 2014; Kambysellis and Heed, 1971). Moreover, ovariole number shows substantial latitudinal and altitudinal clinal variation on different continents, providing further evidence that ovariole number is under selection (Capy et al., 1993; Gibert et al., 2004; Wayne et al., 2005).

The repleta group is one of the largest species group in *Drosophila* genus comprising over 100 species with striking morphological and behavioural diversity (Durando et al., 2000; O'Grady and Markow, 2012; Smith et al., 2012). Particularly interesting is the cactophilic *D. mojavensis*, which has emerged as an important system for understanding the genetic basis of local adaptation, providing significant insights into the early events associated with ecological speciation. *D. mojavensis* is found in four geographically isolated areas in the arid regions of southwestern USA and northwestern Mexico and utilizes necrotic tissues of distinct local host cactus for both feeding and breeding (Figure 4.1). In the mainland Sonoran Desert, *D. mojavensis* uses the organ pipe cactus, *Stenocereus thurberi*, and occasionally shares the columnar cina cactus, *S. alamosensis*, with sister species *D. arizonae.* Although organ pipe cactus is abundant in Baja California, *D. mojavensis* utilizes almost exclusively the pitaya agria cactus, *S. gummosus*, in this region. The population in Mojave Desert uses red barrel cactus, *Ferocactus cylindraceous*, while on Santa Catalina Island, where columnar cacti are absent, the prickly pear cactus species, *Opuntia demissa* and *O. littoralis*, serve as the host (Fellows and Heed, 1972a; Ruiz and Heed, 1988).

The different cacti used by each population differ in a variety of chemical compounds, including free fatty acids and sterols, and in the bacterial and yeast communities that colonize and decompose the cactus tissue into food for these flies (Fogleman and Starmer, 1985; Fogleman et al., 1981; Starmer and Fogleman, 1986). Besides differences in chemical composition, the total size of the plant and their necroses also vary among cactus hosts. Larger cacti, such as the organ pipe cactus, contain stable necroses of large dimensions, whereas in small cacti, such as *Opuntia* sp., necroses are small and ephemeral (Breitmeyer and Markovw, 1998). Each cactus

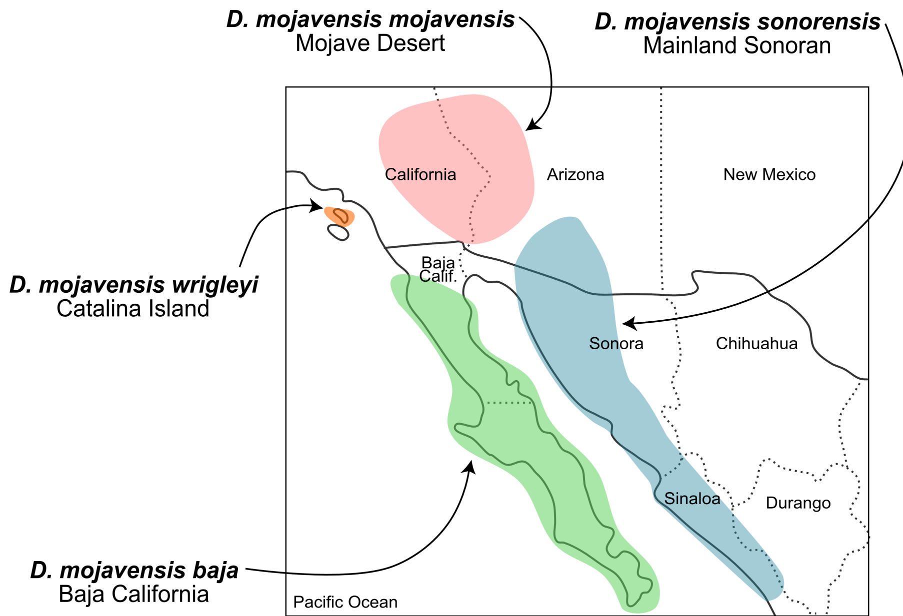


Figure 4.1: Distribution and cactus host use of the four *D. mojavensis* subspecies. Map of the southwestern United States and northwestern Mexico showing the geographical distributions of *D. mojavensis baja*, *D. mojavensis wrigleyi*, *D. mojavensis mojavensis* and *D. mojavensis sonorensis* and their respective host cactus (adapted from (Pfeiler et al., 2009)).

thus offers a distinct ecological niche to the resident flies, which may drive genetic divergence between populations of *D. mojavensis.*

Studies using cytological and molecular data have clearly shown strong genetic differentiation between the four populations. Yet, the evolutionary relationships between them, and which population is ancestral, have proven difficult to resolve (Machado et al., 2007; Reed et al., 2007; Ruiz et al., 1990; Smith et al., 2012). In addition to genetic variation, several physiological, morphological, and behavioural adaptations have been reported between populations, which has led to them being recently described as subspecies: *D. mojavensis baja*, *D. mojavensis sonorensis*, *D. mojavensis mojavensis*, and *D. mojavensis wrigleyi* (Figure 4.1) (Date et al., 2013; Etges et al., 2010; Pfeiler et al., 2009; Richmond et al., 2012).

Previous studies have described that ovariole number shows minor variations (i.e. ovariole number ranges from 25 to 33) among populations

of *D. mojavensis* collected from the field or reared in laboratory cactus (Markow, 1996). I therefore tested whether ovariole number varies between subspecies of *D. mojavensis* reared under standard laboratory conditions. I confirmed that ovariole number varies considerably between the four *D. mojavensis* subspecies. This finding prompted me to focus on two *D. mojavensis* subspecies that display the greatest divergence in ovariole number, *D. moj. sonorensis* and *D. moj. wrigleyi*. I first examined whether differences in ovariole number were associated with changes in three life history traits: female fecundity, female body size, and developmental time. Next, I compared the ovary development in each subspecies to test whether developmental processes known to regulate ovariole number in response to nutrition also underlie differences in ovariole number between the two subspecies. Finally, I investigated how environmental and genetic variation shape ovariole number in the two *D. mojavensis* subspecies.

4.2 Material and Methods

4.2.1 Species stocks

The following species were obtained from the Drosophila Species Stock Center (University of California, San Diego), with the exception of one *D. arizonae* line which was kindly provided by Janelia Farm Research Campus (Ashburn, Virginia): *D. arizonae*, *D. mojavensis baja*, *D. mojavensis mojavensis*, *D. mojavensis sonorensis*, *D. mojavensis wrigleyi* and *D. mulleri* (Table 4.1). Fly stocks were maintained at 22°C in bottles on standard fly food (4.5% molasses, 7.2% sugar, 7% cornmeal, 2% yeast extract, 1% agar and 2.5% Nipagin solution).

4.2.2 Larval staging

Adults were allowed to oviposit for 4-6 hours on fresh plates (60 × 15 mm Petri dish) containing standard fly food. Often, females would lay multiple eggs in very close proximity. When I controlled for overcrowding

Table 4.1: Species stocks used in this study. Stock numbers from Drosophila Stock Center (DSSC) with expection of one *D. arizonae* line from Janelia Farm Research Campus (JFRC).

Species/Subspecies	Stock Number
D. arizonae	JFRC
D. arizonae	15081-1271.29
D. moj. baja	15081-1351.30
D. moj. baja	15081-1352.34
D. moj. mojavensis	15081-1352.00
D. moj. mojavensis	15081-1352.01
D. moj. sonorensis	15081-1352.26
D. moj. sonorensis	15081-1352.32
D. moj. wrigleyi	15081-1352.14
D. mulleri	15081-1371.01
D. mulleri	15081-1379.30

by performing the same protocol as described in Chapters 2 and 3, this would favour bacterial contamination during the first and second larval instar, as larvae tended to aggregate in large groups while foraging and burrowing into a small region of the food plate. As larval density was shown to influence developmental time and thorax length in different populations of *D. mojavensis* (Etges and Heed, 1987), I controlled larval density at several time points throughout larval development to avoid both overcrowding and bacteria contaminations. This was done by cutting the food containing larvae into smaller pieces every other day and gently transferring them to fresh food plates (60 $\times$ 15 mm Petri dish). After egg laying (AEL), plates were maintained at 25°C in a 12 h light-dark cycle with 70% humidity. In these conditions, both *D. moj. sonorensis* and *D. moj wrigleyi* exhibit similar generation times (15-16 days from egg to adult). At day 6 AEL, larvae were selected 0-2 hours after ecdysis to L3 (AL3E) and transferred onto new food plates (40-60 larvae per plate) until the time of dissection [0 h, 20 h, 40 h, 60 h, 80 h AL3E and at pupariation (*D. moj. sonorensis*, 110 h AL3E; *D. moj. wrigleyi*, 104 h AL3E)]. Each time point was replicated at least twice.

4.2.3 Immunocytochemistry, imaging and analysis

Immunocytochemistry was performed as previously described (see Chapter 2). The primary antibody mouse anti-Engrailed (Developmental Studies Hybridoma Bank 4D9, 1:40) was used to identify terminal filament cells (TFCs). The following secondary reagents were used: Alexa 568 (Invitrogen, 1:200) and TRICT-Phalloidin (Sigma, 1:200). Samples were mounted on a poly-L-lysine-coated coverslip using Fluoromount-G (SouthernBiotech) and imaged using a Zeiss LSM 510 Meta confocal microscope. Measurements of total number of forming terminal filaments (TFs) and ovary volume were performed as previously in Chapter 2 and 3 described using ImageJ (NIH) and Adobe Photoshop (Adobe Systems).

4.2.4 Dietary manipulations

Well-fed flies were allowed to deposit eggs overnight on plates containing standard fly food. Egg lays were repeated in two independent days with different parental flies to minimize possible parental effects on larval development. Eggs were collected and groups of 50 eggs were randomly transferred to vials containing one of the following food concentrations: standard fly food (100%), standard fly food diluted with 1% agar in a ratio of 1:2 (50%), 1:4 (25%) or 1:8 (12.5%). The total volume of food was the same in all food concentrations. Four replicates were performed for each food concentration and subspecies. Developing larvae were reared at 25°C in a 12 h light-dark cycle with 70% humidity until all individuals eclosed or died. Eclosed flies were transferred to fresh vials with standard fly food.

4.2.5 Experimental crosses to generate F1 and F2 hybrids

Ten virgin females from *D. moj. wrigleyi* were grouped with five males from *D. moj. sonorensis* in vials on standard food (n=5 vials). The reciprocal cross was also made, but only four replicates laid fertile eggs (n=4 vials). Once females were sexually matured (around 8-10 days of age), they were left to lay eggs in food vials for 24 h. Overcrowding

was controlled as previously mentioned. This procedure was repeated in three independent days to maximize the number of offspring. Eclosed F1 hybrids from each replicate were grouped (ten females and five males per vial) in fresh vials until sexually mature. F1 hybrids were then left to lay eggs using the same procedure as described above. A subset of F1 hybrid females were frozen after 8-10 days for ovariole number counts. A similar protocol was implemented to obtain ovaries from F2 hybrid females.

4.2.6 Measurements of life history traits: developmental time, adult body size, ovariole number and female fecundity

The average time to pupariation was measured by transferring newly ecdysed L3 larvae to food vials (10-20 larvae per vial) and counting the number of larvae pupariating (immobile larvae with evaginated spiracles) every three hours on day 10-11 AEL. To quantify adult body size, pharate adults were individually weighed on a Sartorius SE2 ultramicrobalance. Males of *D. mojavensis* lack sex combs, so I was unable to distinguish males from females at this stage. After weighing, I kept each pharate adult inside an eppendorf until eclosion and females and males were identified based on their external genitalia. Ovariole number was counted for both ovaries from mated females (8-10 days of age) as previously described (see Chapter 2). To determine female fecundity in each subspecies, I implemented two experimental designs: i) three virgin females were grouped with two virgin males (grouped females, n=10 vials/subspecies), and ii) one virgin female was grouped with one virgin male (single females, n=20 vials/subspecies). Flies were transferred to fresh vials every day during the first 20 days after eclosion. The total number of eggs in each vial was counted daily. Variation between biological replicates differed significantly in single females in both subspecies ($p<0.0001$, *D. moj. sonorensis* and *D. moj. wrigleyi*, Kruskal-Wallis).

4.2.7 Statistical Analysis

The distribution of residuals was tested for normality using Q-Q plots and the appropriate statistical test was applied. Differences in ovariole

number among pairs of species were tested with ANOVA followed by Tukey's multiple comparison test. To evaluate differences in total number of eggs laid during 20 days after eclosion, a Poisson regression model (using the R function 'glm') was performed. Correlations between female body size and ovariole number were tested using the Pearson's Correlation coefficient. The distribution of residuals was non-normal for body size and developmental time, and thus, a Wilcoxon rank test was used for pairwise comparisons. Differences in the timing of the onset of TFC differentiation were tested with a Chi-squared test. ANCOVAs were used to evaluate differences in the rates of TF formation and of ovary growth. To test whether survival in four food concentrations was different among subspecies, Kruskall-Wallis rank test was performed. The same test was used to evaluate whether survival within each subspecies differs among food concentrations. To compare the effects of nutrition on ovariole number and female body size between subspecies, ANOVAs were performed. The distribution of residuals for developmental time was non-normal. For this dataset, differences between subspecies were tested using a generalized liner model with Poisson distribution. All data analyses and statistics were conducted using R v3.1.2 (R Development Core Team, 2014). Plots were made using GraphPad Prism v6 (GraphPad Software). Statistical tests and p-values are indicated in the text and figures.

4.3 Results

4.3.1 Ovariole number diversity in species of the *Drosophila mulleri* subgroup

To evaluate the diversity in ovariole number among *D. mojavensis* subspecies, I counted the number of ovarioles in females of each subspecies and of two additional cactophilic species of the repleta group, *D. arizonae* and *D. mulleri*. These two species are closely related to *D. mojavensis*, but utilize different cactus hosts and have broader geographical distributions (Oliveira et al., 2012; Reed et al., 2007). Additionally, I collected data from two independent lines of each species/subspecies, with exception

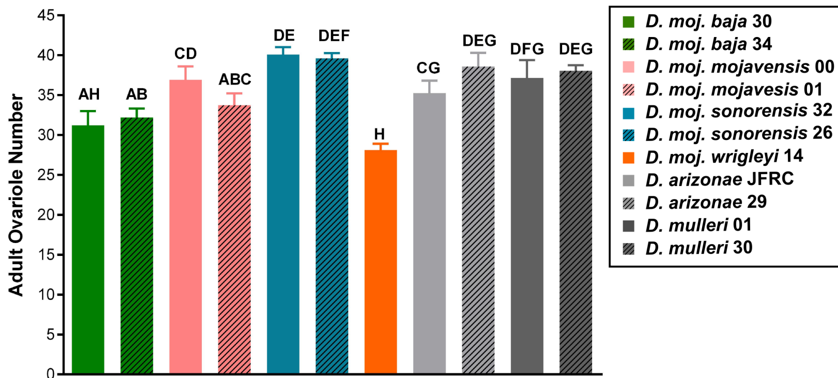


Figure 4.2: Ovariole number in females of *D. mojavensis wrigleyi* is reduced relative to other *D. mojavensis subspecies.* Adult ovariole number in females of *D. moj. baja* (two lines, 15081-1351.30 and 15081-1352.34, n=14 and 27), *D. moj. mojavensis* (two lines, 15081-1352.00 and 15081-1352.01, n=10 and 31), *D. moj. sonorensis* (two lines, 15081-1352.32 and 15081-1352.26, n=70 and n=69) and *D. moj. wrigleyi* (one line 15081-1352.14, 14, n=69). *D. arizonae* (two lines, JFRC and 15081-1271.29, n=17 and 19) and *D. mulleri* (two lines, 15081-1371.01 and 15081-1379.30, n=21 and 21). The numbers in the graph legend represent the last digits from the stock number of each line used. from the Plotted values represent means, and error bars show 95% confidence intervals of the means. One-way ANOVA using Tukey's pairwise test comparison (bars with same letters are not significantly different).

of *D. moj. wrigleyi*, to validate that my measurements of ovariole number were species-specific (Table 4.1). Indeed, the variability between lines in each species/subspecies was not significantly different, ensuring that ovariole number is robust within a given species/subspecies (Figure 4.2). Conversely, ovariole number differed considerably among different members of the repleta group. The island subspecies, *D. moj. wrigleyi*, had the lowest number of ovarioles (28.1±3.3) when compared either to other *D. mojavensis* subspecies (with exception of *D. moj. baja* 15081-1351.30) or to other repleta species, *D. arizonae* and *D. mulleri* (Figure 4.2). On the other hand, the subspecies inhabiting the Sonoran Desert, *D. moj. sonorensis*, showed the highest number of ovarioles among *D. mojavensis* subspecies (39.6±2.8 in *D. moj. sonorensis* 15081-1352.26 and 40.1±3.8 in *D. moj. sonorensis* 15081-1352.32). Both *D. arizonae* (only line 15081-1271.29) and *D. mulleri* showed a similar ovariole number as both of the *D. moj. sonorensis* lines and *D. moj. mojavensis* line 15081-1352.00. Based on these observations, I focused on two *D. mojavensis*

subspecies that display the greatest divergence in ovariole number, *D. moj. wrigleyi* and *D. moj. sonorensis* 15081-1352.32 (henceforth referred as *D. moj. sonorensis* and *D. moj. wrigleyi*).

4.3.2 Divergence of life history traits in *D. moj. sonorensis* and *D. moj. wrigleyi*

Female fecundity

Studies in several species of the *melanogaster* group demonstrated that the number of ovarioles correlates positively with the number of eggs females lay (see Chapter 2) (Klepsatel et al., 2013b,a; R' kha et al., 1997; Kambysellis and Heed, 1971). I therefore asked whether females of *D. moj. sonorensis*, which have a higher number of ovarioles, lay significantly more eggs than *D. moj. wrigleyi* females. Preliminary observations revealed that flies of *D. moj. sonorensis* tend to aggregate in one side of the food plate and lay multiple eggs in a small region of the food plate. This oviposition behaviour was also observed in flies of *D. moj. wrigleyi*, although it was less conspicuous. Hence, to fully investigate whether female fecundity differs between the two *D. mojavensis* subspecies, I counted the number of eggs laid in each subspecies in two experimental groups: grouped females (three females and two males) and single females (one female and one male).

The dynamics of egg laying in grouped females were erratic in both *D. mojavensis* subspecies, yet an interesting pattern emerged (Figure 4.3A). Eggs were laid in clutches in given days, with almost no eggs laid in the intervening days. This irregular pattern of egg laying was more noticeable in single females (Figure 4.3B). In two biological replicates, in particular, individual females laid more than one hundred eggs in a single day and very few eggs were oviposited in the remaining days (higher peaks in light blue in Figure 4.3B). Even though the dynamics of egg laying was similar in the two *D. mojavensis* subspecies, the total number of eggs laid over 20 days was significantly lower in *D. moj. wrigleyi* relative to *D. moj. sonorensis*, and this was seen in both grouped and single females ($p<0.0001$, Poisson regression model) (Figure 4.3C, D).

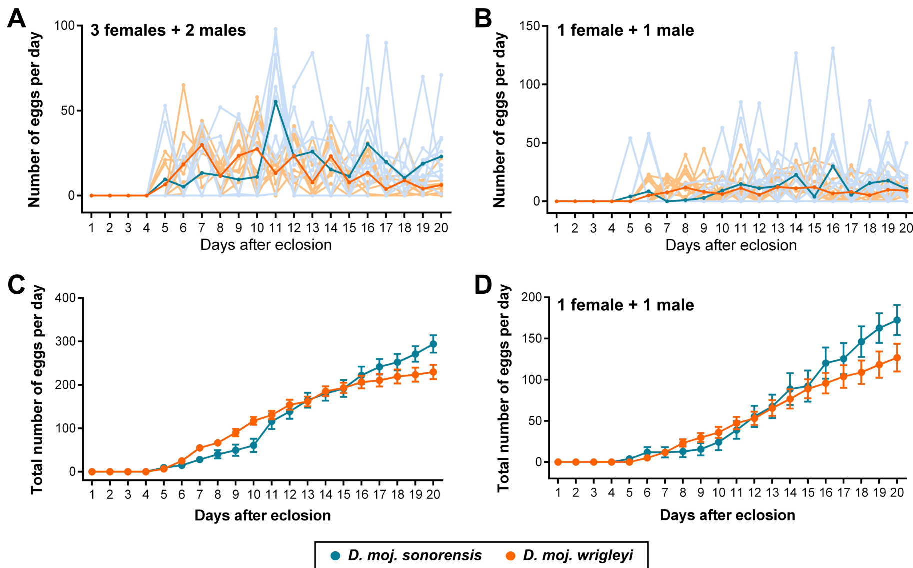


Figure 4.3: Daily egg production in *D. moj. sonorensis* and *D. moj. wrigleyi*. Number of eggs laid was counted daily in two experimental groups: (A, C) grouped females (three females and two males) and (B, D) single females (one female and one male). (A, B) Mean number of eggs laid in *D. moj. sonorensis* (dark blue line) and *D. moj. wrigleyi* (dark orange line) in the first 20 days after eclosion. Each biological replicate is shown in light coloured lines. (C, D) The data from (A, B) plotted as cumulative sum of the mean number of eggs laid in *D. mojavensis sonorensis* (dark blue line) and *D. mojavensis wrigleyi* (dark orange line). Error bars show standard error of the means.

Female body size

Body size often plays an important role in insect reproductive success and there is a correlation between body size and ovariole number in many *Drosophila* species (Bergland et al., 2008; Green and Extavour, 2012; Hodin and Riddiford, 2000; Kambysellis and Heed, 1971; Robertson, 1956; Santos et al., 1992; Wayne et al., 1997). To examine whether *D. moj. sonorensis* females were larger than females of *D. moj. wrigleyi*, I weighed pharate adults as a proxy of adult body size. Indeed, *D. moj. sonorensis* had significantly larger body sizes than *D. moj. wrigleyi*, and this increase in body size was seen in both sexes (Figure 4.4A). These differences in female body size positively correlated with differences in ovariole number ($p<0.0001$, $R=0.80$, Pearson's correlation coefficient).

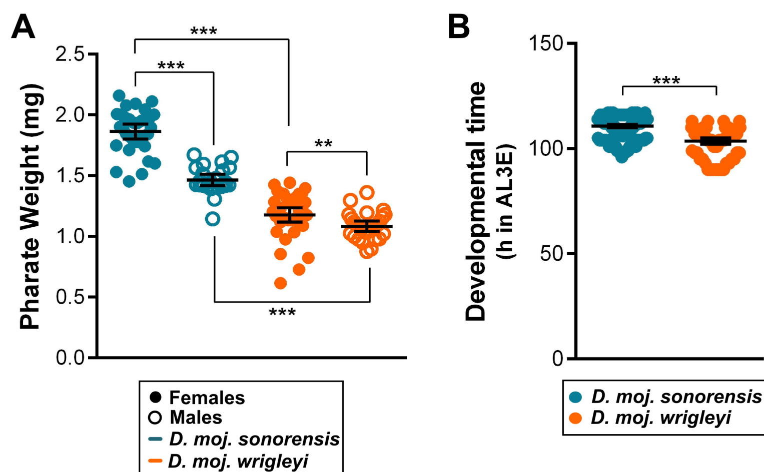


Figure 4.4: Adult body size and duration of L3 development in *D. moj. sonorensis* and *D. moj. wrigleyi*. (A) Mean pharate weight of males (open points) and females (closed points) of *D. moj. sonorensis* (blue points) and *D. moj. wrigleyi* (orange points). $n \geq 20$ pharate adults for both sex/subspecies. (B) Mean developmental times represented in hours after third instar ecdysis (h AL3E) to pupariation in *D. moj. sonorensis* (blue points) and *D. moj. wrigleyi* (orange points). $n \geq 100$ larvae for both subspecies. Error bars show 95% confidence intervals of means. Wilcoxon rank test:**$p<0.01$, ***$p<0.001$.

Developmental time

The formation of ovarioles occurs during the third and final instar (L3) larval and early pupal stages in a temporally and spatially controlled

fashion (Kerkis, 1931; King, 1970; King et al., 1968). The number of TFs present at the onset of metamorphosis determines adult ovariole number (Hodin and Riddiford, 2000; Sarikaya et al., 2012; Sarikaya and Extavour, 2015). Therefore, differences in the duration of L3 larval stages could explain the divergence in ovariole number between *D. moj. sonorensis* and *D. moj. wrigleyi*. To test this hypothesis, I measured the time it takes for newly ecdysed L3 larvae of each subspecies to reach pupariation. I found that *D. moj. sonorensis* larvae pupariated on average at 111 h after L3 ecdysis (AL3E), while larvae of *D. moj. wrigleyi* had a shorter developmental time, pupariating on average at 104 h AL3E (Figure 4.4B). Thus, the difference in ovariole number could be due to shorter L3 duration in *D. moj. wrigleyi*. If this were the sole determinant of the difference in the ovariole number, than I would expect that the timing of TFC differentiation, the rate of TF formation, and rate of ovary growth would be the same between the two subspecies.

4.3.3 The dynamics of TF formation and ovary growth during L3 larval stages

I next analysed the ovary development in both subspecies to identify if one or all of the three developmental processes – the onset of TFC differentiation, the rate of TF formation and of ovary growth – differ among the two subspecies. The onset of TFC differentiation is easily identified by the upregulation of the transcription factor Engrailed (En) specifically in the TFCs (Patel et al., 1989). While TFCs and few short TFs were visible at 40 h AL3E in most, if not all, ovaries from *D. moj. sonorensis*, I detected TFCs in only a few ovaries from *D. moj. wrigleyi* larvae (Figure-4.5C, I, M, N). This difference in the timing of the onset of TFC differentiation was significant ($p<0.0001$, $\chi^2 = 39$, $df = 1$, Chi-Square Test). At 60 h AL3E, all ovaries from both subspecies showed TFCs and at least a few TFs (Figure 4.5D, J). Even though the onset of TFC differentiation was delayed in *D. moj. wrigleyi*, the rate at which new TFs were formed between 40 h and 80 h AL3E was similar to *D. moj. sonorensis* ($p=0.3457$, ANCOVA) (Figure 4.5N). In the hours preceding pupariation, the rate of TF formation in *D. moj.*

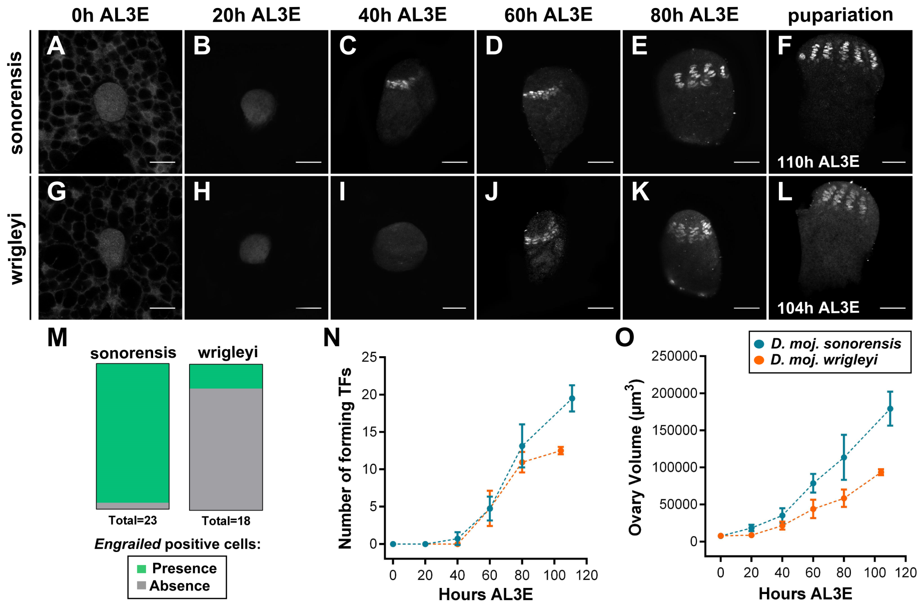


Figure 4.5: The dynamics of TF formation and ovary growth in *D. moj. sonorensis* and *D. moj. wrigleyi.* (A-L) Shown is terminal filaments (TFs) marked with En (grey) in ovaries from larvae of (A-F) *D. moj. sonorensis* and (G-L) *D. moj. wrigleyi.* Scale bar: 20µm. (M) Proportion of ovaries with presence (green) and absence (grey) of En-positive cells. Total number of ovaries analysed is shown for each subspecies. (N) Number of forming terminal filaments (TFs) and (O) ovary volume of ovaries from larvae of *D. moj. sonorensis* (blue points) and *D. moj. wrigleyi* (orange points). Plotted values represent means, and error bars show standard error of the means. In some cases, error bars are too small to be seen. L3: third instar larvae; AL3E: after L3 ecdysis.

wrigleyi decreased substantially when compared to *D. moj. sonorensis* ($p<0.05$, ANCOVA) (Figure 4.5N). Finally, the rate of ovary growth was significantly different between the two subspecies throughout L3 development ($p<0.0001$, ANCOVA). (Figure 4.5O).

4.3.4 The effects of larval nutrition in developmental time, female body size, and ovariole number

In *D. melanogaster*, ovariole number varies in response to larval nutrition (see Chapter 2) (Bergland et al., 2008; Green and Extavour, 2014; Hodin and Riddiford, 2000; Sarikaya et al., 2012; Tu and Tatar, 2003). Nutritional plasticity in ovariole number within a given species was

recently shown to be linked with ecological specialization; that is, generalist species show high nutritional plasticity compared to specialist species (Green and Extavour, 2014). As *D. moj. sonorensis* and *D. moj. wrigleyi* larvae develop in distinct nutritional environments, I therefore examined the nutritional plasticity in ovariole number of both subspecies by rearing individuals from egg to adult eclosion either in standard fly food (100%) or in food diluted to 50%, 25% or 12.5% its original concentration. Differences in nutritional plasticity were estimated by comparing the slopes of the reactions norms between the two subspecies. Additionally, I analysed the nutritional plasticity in female body size and developmental time (from egg to pupae) in both subspecies, as these life history traits also show remarkable plasticity in response to larval nutrition (Mirth and Shingleton, 2012).

As expected, survival to pupation in both subspecies was strongly reduced with decreased food concentration ($p<0.0001$, Kruskal-Wallis rank sum test) (Figure 4.6A). Nevertheless, I did not observe any differences in survival in response to nutrition between subspecies ($p=0.9099$, Kruskal-Wallis rank sum test) (Figure 4.6A). Developmental time, female body size, and ovariole number were significantly affected by larval nutrition in both subspecies; larvae reared in lower food concentrations took longer to develop and showed considerable reductions in both female body size and ovariole number when compared to larvae fed on higher food concentrations (Figure 4.6B-D and Tables 4.2, Table 4.3 and Table 4.4). Furthermore, genetic background contributed significantly for all traits analysed (Tables 4.2, Table 4.3 and Table 4.4). However, the slopes of the reaction norms, hence nutritional plasticity, only differ for female body size (Tables 4.2, Table 4.3 and Table 4.4). Taken together, these findings suggest that while both the genotype and the environment contribute to variation in developmental time, body size, and ovariole number, genetic variation in plasticity is only apparent for body size.

4.3.5 Phenotypic analysis of F1 and F2 hybrids

In the past decades, quantitative genetic studies have identified numerous loci that account for variation in ovariole number within and between

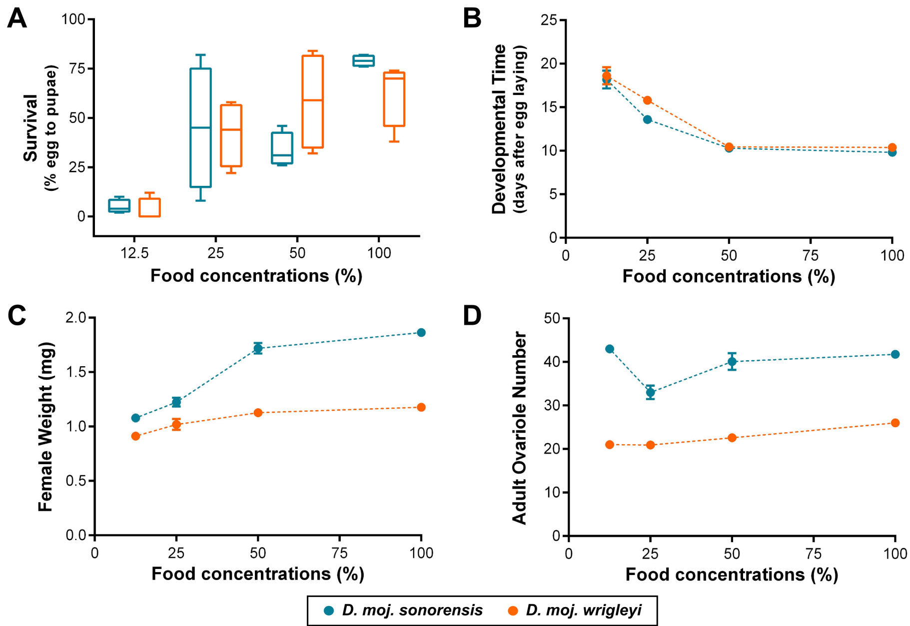


Figure 4.6: The effects of larval nutrition in developmental time, female body size and ovariole number in *D. moj. sonorensis* and *D. moj. wrigleyi.* (A) Average survival to pupation in *D. moj. sonorensis* (blue boxplots) and *D. moj wrigleyi* (orange boxplots) larvae reared in one of four food concentrations: 100%, 50%, 25% and 12.5%.(B) Developmental time, (C) female weight and (D) adult ovariole number for *D. moj. sonorensis* (blue points) and *D. moj wrigleyi* (orange points) larvae reared in one of four food concentrations: 100%, 50%, 25% and 12.5%. Plotted values represent means and error bars show standard error of means. In some cases, error bars are too small to be seen.

Table 4.2: Generalized linear model (family=Poisson) for developmental time.

Source of variation	df	Deviance	*p*-value
Subspecies	1	13.984	0.0002 ***
Food concentration	1	183.469	<0.0001 ***
Subspecies x Food concentration	1	1.817	0.1777 ns
Error	663	-	-

Table 4.3: Two-away ANOVA model for female body size.

Source of variation	df	*F*	*p*-value
Subspecies	1	216.554	<0.0001***
Food concentration	1	64.017	<0.0001***
Subspecies x Food concentration	1	14.617	<0.0001***
Error	165	-	-

Table 4.4: Two-away ANOVA model for ovariole number.

Source of variation	df	*F*	*p*-value
Subspecies	1	335.4036	<0.0001 ***
Food concentration	1	29.5408	<0.0001 ***
Subspecies x Food concentration	1	0.4847	0.6966 ns
Error	74	-	-

species of the *melanogaster* group (Bergland et al., 2008; Orgogozo et al., 2006; Wayne and McIntyre, 2002; Wayne et al., 2001). However, only a small number of candidate genes have been identified and functionally investigated (Green and Extavour, 2012, 2014; Orgogozo et al., 2006). Green and Extavour (2012) advocate that the identification of relevant candidate genes will benefit from a better understanding of the developmental mechanisms that generate diversity in ovariole number (Green and Extavour, 2012). Throughout this thesis, I extensively investigated the developmental mechanisms that contribute to variation in ovariole number. In this final section, I show my preliminary exploration of the genetic basis of intraspecific variation in ovariole number and female body size.

For this purpose, I examined ovariole number and female body size in F1 and F2 hybrids between *D. moj. sonorensis* and *D. moj. wrigleyi*. In both reciprocal crosses, F1 hybrids showed intermediate values in ovariole number, suggesting that variation in ovariole number involves alleles with opposite dominant effects or without any dominance at all (Figure 4.7A, B). Female body size in F1 hybrids was skewed towards the smaller parental line, *D. moj. wrigleyi* (Figure 4.7C, D), which may indicate that alleles involved in regulating smaller body size are dominant over those that determine larger body size. However, even though larval density was controlled as carefully as possible, the number of larvae in each bottle varied substantially, which could contribute to a reduction in female body size in F1 hybrids. Thus, I cannot exclude the possibility that the smaller adult size of F1 hybrids is a consequence of the larval rearing environment.

Interestingly, in the F2 hybrids the variance for ovariole number and female body size was similar to that observed in the F1 hybrids (Figure 4.7).

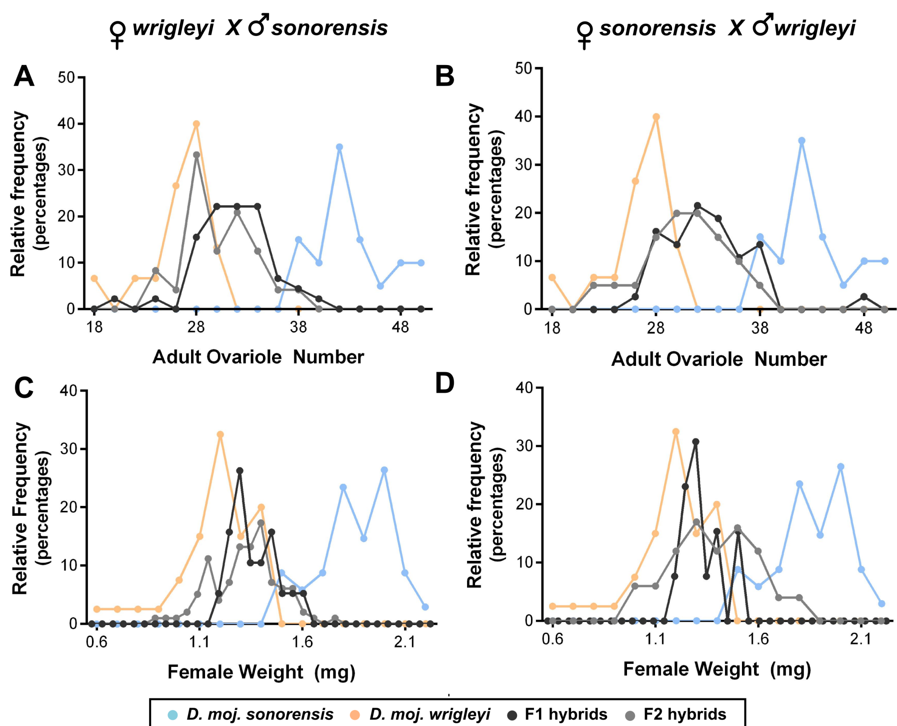


Figure 4.7: Ovariole number and female body size in F1 and F2 hybrids. Relative frequency distribution (in percentages) of ovariole number (n=20-45) (A, B) and female body size (n=20-90) (C, D) in the parental lines, *D. moj. sonorensis* (light blue line) and *D. moj. wrigleyi* (light orange line), and the F1 (black line) and F2 hybrids (grey line) from both reciprocal crosses between *D. moj. sonorensis* and *D. moj. wrigleyi*.

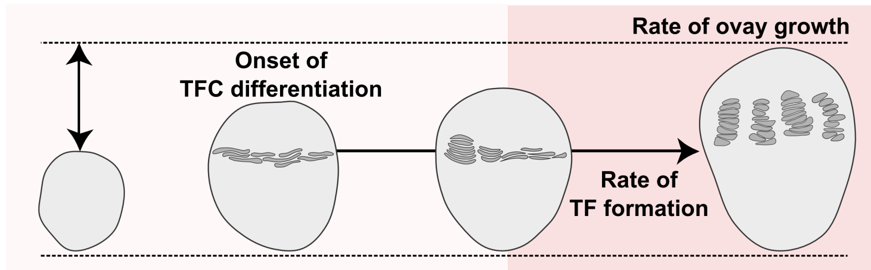
Thus, it is likely that few loci contribute to divergence in ovariole number and female body size between *D. moj. sonorensis* and *D. moj. wrigleyi*. More generations would be required to further break up possible linkage associations and obtain a broader range of phenotypes.

4.4 Discussion

4.4.1 Plastic responses and evolved variation in ovariole number

By examining the ovary development in both subspecies, I found that all three developmental processes differ between *D. moj. sonorensis* and *D. moj. wrigleyi* (Figure 4.8A). The onset of TFC differentiation was delayed in *D. moj. wrigleyi* relative to *D. moj. sonorensis*. Such delay is not due to differences in developmental time between subspecies, since *D. moj. wrigleyi* larvae are the ones that develop faster. Even though the timing of the onset of TFC differentiation was different between species, the rate of TF formation was reduced only at the end of L3 development in *D. moj. wrigleyi* when compared with *D. moj. sonorensis*. This contrasts with the nutritional-induced differences during ovary development (Figure 4.8B) (see Chapter 2). Starving pre-critical weight larvae delays the onset of TFC differentiation and reduces the rate of TF formation throughout the post-critical weight phase of development. This correlation is further observed when either insulin/insulin-like growth signalling (IIS) or ecdysone signalling pathways are suppressed in the larval ovary of well-fed larvae (see Chapter 3).

How is it that changes in the onset of TFC differentiation have no effect on the rate of TF formation between subspecies? A possible explanation is that once the first TFCs differentiate from the surrounding somatic cells in *D. moj. wrigleyi*, they rapidly intercalate into stacks and hence the number of forming TFs quickly increases. Additionally, the relative number of TFCs that initially emerged at the onset of TFC differentiation may also differ between subspecies. Perhaps at the onset of TFC differentiation, a higher relative number of TFCs is produced in *D. moj. wrigleyi* ovaries,



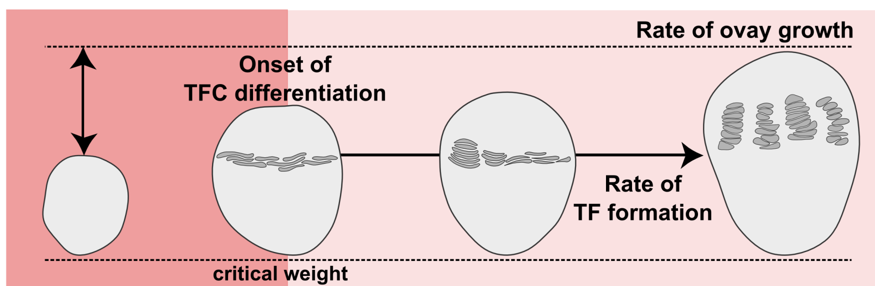


Figure 4.8: Changes in distinct developmental processes underlie nutritional-induced and subspecies-specific variation in ovariole number. (A) Changes in the rates of TF formation and of ovary growth are responsible for ovariole number divergence between *D. mojavensis* subspecies. (B) Before critical weight, starvation delays the onset of TFC differentiation and arrests ovary growth. In contrast, the rates of TF formation and of ovary growth continue when post-critical weight larvae are poorly fed. However, these rates are considerably reduced, resulting in fewer ovarioles than well-fed larvae.

which could contribute to a faster formation of new TFs in a short period of time.

Even though the rate of TF formation is initially similar, at the end of L3 development the rate of TF formation is reduced, contributing to the divergence in ovariole number between *D. moj. sonorensis* and *D. moj. wrigleyi* (Figure 4.8A). Changes in the rate of TF formation were also observed when post-critical weight larvae were poorly fed (Figure 4.8B) (see Chapter 2). In addition, Hodin and Riddiford (2000) found that rearing *D. melanogaster* larvae on diluted medium only reduced the rate of TF formation at the end of the L3, similar to what I observed in *D.*

moj. wrigleyi.

The rate of ovary growth was considerably reduced in *D. moj. wrigelyi* when compared with *D. moj. sonorensis*. The total number of TFs greatly depends on the number of TFCs that differentiate from the somatic cell pool (Sarikaya et al., 2012). Therefore, a slower rate of ovary growth either in *D. moj. wrigleyi* or in starved post-critical weight larvae may gradually constrain the number of cells available to form TFCs, thereby reducing the rate of TF formation. Taken together, I propose that ovary growth rates have diverged between *D. moj. wrigleyi* and *D. moj. sonorensis*, generating differences in ovariole number by limiting the number of somatic cells available to form TFCs.

In a previous study by Hodin and Riddiford (2000), the authors concluded that nutritionally-induced variation in ovariole number is restricted to alterations in the rate of TF formation, while changes in a broader range of developmental processes can account for variation in ovariole number both between populations and between species (Hodin and Riddiford, 2000). My results differ from this study in several ways. First, to investigate the developmental processes that account for nutritional-induced differences, I reared L3 larvae in a medium containing only sugar and agar, which significantly reduced both ovariole number and body size and delayed developmental time. Under these conditions, the onset of TFC differentiation, the rate of TF formation and the rate of ovary growth were all affected. In contrast, Hodin and Riddiford (2000) reared larvae from egg to adult in 50% diluted food from its original concentration in order to avoid any change in body size and developmental time (Hodin and Riddiford, 2000). Such diluted food provides a less stressful environment for larvae to develop, and only altered the rate of TF formation at the end of larval development. Taken together, the range of developmental processes altered by nutrition depends partly on the severity of the nutritional conditions.

Second, my analysis was performed with significant resolution throughout L3 larval development, which allowed me to detect differences in the onset of TFC differentiation, the rate of TF formation, and the rate of ovary growth between *D. mojavensis* subspecies and between nutritional

responses. Hodin and Riddiford (2000), by contrast, inferred differences in the onset of TFC differentiation by counting the number of TFs at 24 h AL3E without any analysis on previous time points (Hodin and Riddiford, 2000). Moreover, the rates of TF formation were only compared during the wandering stage (i.e. when larvae leave the food to find a pupariation site) and no comparisons were performed amongst rates of ovary growth (Hodin and Riddiford, 2000).

Third, Hodin and Riddiford (2000) compared five species in the melanogaster subgroup with a much greater range of ovary sizes than those studied here (Hodin and Riddiford, 2000). *D. sechellia* has severely reduced ovariole number relative to *D. melanogaster* (R' kha et al., 1997; Orgogozo et al., 2006; Hodin and Riddiford, 2000; Green and Extavour, 2014). These differences in ovariole number are due to a smaller size of ovarian primordium and slower rates of TF formation in *D. sechellia*(Green and Extavour, 2012; Hodin and Riddiford, 2000). Hence, the difference in ovariole number between these two species is defined by changes in ovary development since embriogenesis. In my study, I focused on two subspecies of *D. mojavensis* with more moderate differences in ovary size. Here, I found that ovary size is indistinguishable between the two races at the onset of the L3. Differences in ovary development, including ovary growth rates and the rate of TF formation, arise only in the final larval stage. Thus, it would seem that much like for the plastic response, the number of developmental processes underlying intra/interspecific genetic variation depends on the magnitude of the difference in ovary size.

Although differences in ovariole number both across species and across populations often correlate with observed plastic responses, these are not the only mechanisms known to generate variation in ovariole number. Green and Extavour (2012) found that differences in ovariole number between two strains of *D. melanogaster* resulted not from differences in ovary growth but from differences in the allocation of somatic cells, which have the potential to become TFC, to different somatic cell fates (Green and Extavour, 2012). In strains with lower ovariole numbers, a greater proportion of the somatic cells adopted a swarm cell fate than in strains

with higher ovariole numbers. Whether this developmental difference corresponds to a plastic response to another type of environmental cue, such as temperature or hypoxia, remains to be discovered.

4.4.2 The relationship between ovariole number and female body size

I have shown that variation in ovariole number between the two *D. mojavensis* subspecies is positively correlated with variation in female body size. However, the relationship between ovariole number and female body size is not always clear (Bergland et al., 2008; Green and Extavour, 2012; Hodin and Riddiford, 2000; Kambysellis and Heed, 1971; Robertson, 1956; Santos et al., 1992; Wayne et al., 1997). The strength of this relationship appears to vary with environmental factors, including nutrient availability and larval competition (Bergland et al., 2008; Kambysellis and Heed, 1971; Santos et al., 1992). For instance, in some specialist species, such as the cactophilic *D. buzzatii* and the Hawaiian *D. mimica*, a positive correlation between thorax length (a proxy index for body size) and ovariole number is found in wild caught flies, but not in laboratory-reared flies (Kambysellis and Heed, 1971; Santos et al., 1992). Furthermore, larvae of *D. melanogaster* reared in low yeast concentrations show a strong positive correlation between thorax length and ovariole number. This correlation is lost when larvae are fed on optimal nutritional conditions (Bergland et al., 2008; Wayne et al., 1997). Perhaps the most interesting case is that of temperature-induced differences in ovariole number and body size in populations of *D. melanogaster*. Larvae reared at higher temperatures produce smaller adults with reduced ovariole number. In contrast, adult from larvae grown at lower temperatures have larger body size, but similar reduction in ovariole number (Klepsatel et al., 2013a; Mirth and Shingleton, 2012). Future studies examining more species and a greater range of environmental conditions will lead to vast improvements in our understanding of the relationship between ovariole number and female body size.

4.4.3 Generating a hypothesis on egg laying behaviour

Ovariole number is an important determinant of female reproductive capacity; females with higher number of ovarioles can potentially lay more eggs (see Chapter 2) (Boulétreau-Merle et al., 1982; Klepsatel et al., 2013b; R' kha et al., 1997). Indeed, *D. moj. sonorensis* females have a greater reproductive capacity than females of *D. moj. wrigleyi*. However, this difference in total number of eggs laid among the two subspecies of *D. mojavensis* only became apparent in the last days of the analysis, suggesting that other factors, such as rate of oogenesis or egg retention, influence female reproductive output. Moreover, substantial differences in male reproductive traits could also exist between the two *D. mojavensis* subspecies, which might contribute to the observed differences in the total number of eggs laid.

Interestingly, both subspecies exhibited an erratic dynamic of egg laying, in which numerous eggs were laid in given days. The rate of egg production may offer a possible explanation for this egg laying behaviour. Ovaries can be defined as synchronous when all ovarioles have at least one mature egg (Kambysellis and Heed, 1971). One such example is found in *D. mulleri*, in which mature eggs develop synchronously and are oviposited in large clutches in a single day (Markow and O'Grady, 2008). A close examination of the number of eggs per ovariole may elucidate whether the observed egg laying behaviour in *D. mojavensis* subspecies is a direct result of a synchronous ovary.

Alternatively, the abundance and distribution of suitable oviposition sites may influence the frequency of oviposition (Kambysellis and Heed, 1971). Females of *D. mojavensis* oviposit in necrotic cacti, which offers a moist and nutritious environment for larval development (Fellows and Heed, 1972b; Ruiz and Heed, 1988). The patchy distribution of cactus hosts and the ephemeral nature of necroses require that *D. mojavensis* efficiently disperse great distances to find the appropriate breeding site (Breitmeyer and Markovw, 1998; Pfeiler and Markow, 2011). In their natural setting, *D. mojavensis* females may retain mature eggs until a potential oviposition site is found, which might lead to an irregular egg laying behaviour. However, if postponing oviposition is advantageous when

suitable resources are infrequently encountered, why is this behaviour maintained in laboratory conditions? The physiological mechanisms regulating the rate of egg laying could be genetically fixed and thus, even in the presence of abundant food resources, the dynamics of egg laying is unchanged.

Finally, the standard fly food provided in laboratory conditions might not be optimal for *D. mojavensis* species. Although *D. mojavensis* is relatively easy to maintain under laboratory conditions, the necrotic cacti provides a complex nutritious environment with specific olfactory cues (Date et al., 2013) that may influence the frequency of oviposition. Further studies using extracts of necrotic cactus could provide new insights on egg laying behaviour in *D. mojavensis* subspecies.

4.5 Conclusions

The aim of this chapter was to elucidate the developmental mechanisms underlying variation in ovariole number between subspecies of *Drosophila mojavensis*. Coupled with the extensive studies regarding their ecology, *D. mojavensis* subspecies represent an attractive model to address the early events leading to evolutionary diversification in ovariole number. Importantly, the results described in this chapter open up new avenues of research in diverse disciplines, including ecology (e.g. what underlies irregular egg laying behaviour?), cellular biology (e.g. what are the underlying mechanisms regulating proliferation, differentiation and intercalation of TFCs) and physiology (e.g. how different traits respond differently to nutrition?).

Acknowledgments

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5

GENERAL DISCUSSION

"And that our greatest accomplishments cannot be behind us, because our destiny lies above us."

– from the film *Interstellar* (2014)

In this thesis, I explored the mechanisms through which the environment can generate diversity in ovariole number within and between *Drosophila* species. I began by characterizing in great detail the effects of larval nutrition on ovariole number in *D. melanogaster* (Chapter 2). This first analysis allowed me to identify two phases of sensitivity during which distinct developmental processes are modified by nutrition. I then demonstrated how hormonal pathways regulate the nutritional response of each developmental process (Chapter 3). Finally, I found that similar developmental changes underlie variation in ovariole number between two subspecies of *D. mojavensis* (Chapter 4). In this final chapter, I will summarize the main contributions of my thesis work and propose future avenues of research that could enhance our understanding of how the environment generates morphological diversity.

5.1 Ovariole number shows distinct phases of nutritional sensitivity

Myriad reports have shown that ovariole number varies greatly in response to variation in nutritional conditions (Bergland et al., 2008; Green and Extavour, 2014; Hodin and Riddiford, 2000; Sarikaya et al., 2012; Tu and Tatar, 2003). However, these studies have not addressed whether critical periods of environmental sensitivity influence the plastic response of ovariole number nor explored whether the developmental processes underlying such variation in ovariole number show different sensitivities to nutrition. The work presented in this thesis is the first study to identify two phases of sensitivity to nutrition that regulate plasticity in ovariole number. Each phase is associated with changes in distinct development processes. In the first phase, starvation delays the onset of TFC differentiation and arrests ovary growth, which drastically reduces ovariole number. On the other hand, changes in nutrition in the second phase have a more modest effect on ovariole number; starving larvae in this phase reduces the rates of TF formation and of ovary growth. Thus, the extent of nutritional plasticity in ovariole number greatly depends on when nutritional variation is experienced during larval development,

highlighting the relevance of critical periods of environmental sensitivity in determining the outcome of plastic responses.

Even though critical periods of environmental sensitivity have been identified in other insect species (Bear and Monteiro, 2013; Kooi and Brakefield, 1999; Nijhout, 2003b; Noor et al., 2008; Schrempf and Heinze, 2006), the mechanisms regulating the timing of such critical periods have been less studied. My work represents one of the first attempts to address this issue. I found that critical weight separates the two phases of sensitivity and presumably reprograms the developing ovary's response to nutritional variation. This finding corroborates previous studies on wing disc development (Mirth et al., 2009; Shingleton et al., 2008). I further provided significant insights into the relative roles of IIS and ecdysone signalling in regulating ovariole number in response to nutrition. Importantly, I found that IIS and ecdysone signalling interact considerably to regulate the three nutrition-sensitive developmental processes.

Many other environmental factors are known to influence ovariole number (Hodin, 2009). For instance, rearing temperature affects ovariole number in natural and laboratory populations of *D. melanogaster* (Delpuech et al., 2011; Hodin and Riddiford, 2000; Klepsatel et al., 2013a; Sarikaya et al., 2012). Moreover, ovariole number shows altitudinal and latitudinal variation within species of the *melanogaster* group; tropical populations have higher ovariole numbers than temperate populations (Boulétreau-Merle et al., 1982; Capy et al., 1993; Delpuech et al., 2011; Klepsatel et al., 2013a). However, temperature and nutrition may modulate ovariole number through different mechanisms. Larvae of *D. melanogaster* reared in lower temperatures have reduced ovariole number. Such reduction is due, at least in part, to an increase in the number of TFCs per TF without any change in total number of TFCs, resulting in the formation of fewer TFs (Sarikaya et al., 2012). Therefore, changes in the intercalation of TFCs during TF formation underlie temperature-induced differences in ovariole number, although it remains unclear if differences in the onset of TFC differentiation or the rate of TF formation might also play a role. Furthermore, the authors have not explored whether TFC intercalation responds to temperature at specific stages in development

nor characterized the molecular mechanisms through which temperature affects TFC intercalation.

Ovariole number is also reduced when *D. melanogaster* are reared at higher rearing temperatures (Hodin and Riddiford, 2000). However, the mechanisms underlying reduced ovariole number at higher temperatures are not known. Interestingly, natural variation in InR alleles correlates with clinal variation in ovariole number (Paaby et al., 2010). Because I have found that IIS regulates nutritional plasticity in the ovary, there might be temperature ranges in which the mechanisms regulating the plastic responses to nutrition and temperature overlap. A similar approach to the one I used in this thesis may help uncover how temperature, and many other environmental factors, modify ovariole number.

5.2 A hypothesis for variation in the onset of TFC differentiation

When larvae are poorly fed before critical weight, both the timing of the small ecdysone peak (Koyama et al., 2014) and the onset of TFC differentiation are delayed. Partially activating ecdysone signalling in the larval ovary results in a precocious onset of TFC differentiation in poorly-fed larvae, confirming that ecdysone signalling is indeed required to induce the onset of TFC differentiation. Furthermore, it shows that when it is not bound to ecdysone, EcR acts as a repressor of TFC differentiation.

Upregulating IIS is also sufficient to trigger a precocious onset of TFC differentiation in malnourished larvae. How can IIS induce the onset of TFC differentiation when ecdysone levels are low? Based on my findings, I propose that IIS might regulate the threshold amount of ecdysone necessary to induce the onset of TFC differentiation. In this scenario, high levels of IIS activity in the larval ovary reduce the threshold of ecdysone sensitivity and thus, TFC precursors might become sensitive to basal levels of ecdysone (Figure 5.1). The onset of TFC differentiation is, therefore, induced even when nutrition and ecdysone titres are low (Figure 5.1B).

Two issues arise from this hypothesis. First, if high levels of IIS activity allow TFC precursors to respond to basal levels of ecdysone, then how

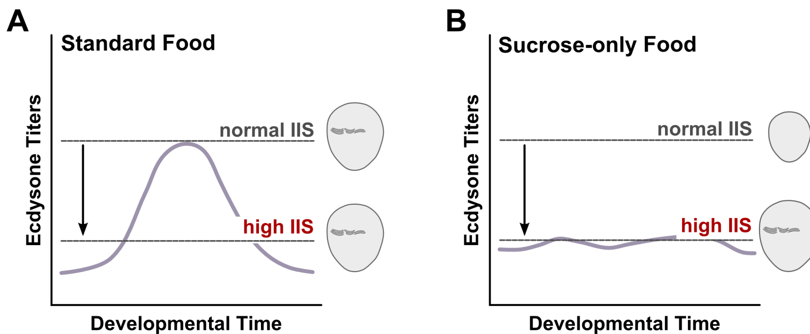

Figure 5.1: A threshold of ecdysone sensitivity controlled by IIS. (A) High levels of IIS activity in the larval ovary might reduce the threshold of ecdysone sensitivity, allowing TFCs to become sensitive to low levels of ecdysone. (B) Under poor nutritional conditions, ecdysone levels are low. Upregulating IIS in the ovary of poorly-fed larvae is sufficient to induce the onset of TFC differentiation, because TFCs are able to respond to basal levels of ecdysone.

come TFC differentiation only occurs between 10 and 15 h AL3E in these ovaries? Second, if the onset of TFC differentiation depends on the activity of ecdysone signalling, why doesn't partially activating this pathway in the ovaries result in precocious onset in fed conditions? The existence of a specific period in development (henceforth referred as responsive period [1] during which TFC precursors are able to respond to levels of ecdysone may offer a plausible explanation that solves both issues. This responsive period appears to overlap with the attainment of critical weight, as the onset of TFC differentiation is likely triggered by the small peak of ecdysone at critical weight. However, while critical weight is nutritional-sensitive, the responsive period is likely robust to nutritional variation, as the onset of TFC differentiation can only be induced around 15 h AL3E independently of the nutritional status of the larvae.

Nevertheless, the timing of such responsive period can be altered. When both pathways are simultaneously upregulated in the larval ovary, TFCs are observed in ovaries from larvae staged at 5 h AL3E, suggesting that the responsive period occurs early in these larvae. However, the mechanisms

[1] Responsive periods are not the same as the critical periods of environmental sensitivity that I described previously. According to Nijhout (2003), environmental cues are integrated during critical periods of environmental sensitivity. Such environmental integration induces changes in hormonal mechanisms. Target tissues only respond to those changes during hormone sensitive periods or responsive periods (Nijhout, 2003b)

underlying this change in the timing of the responsive period are difficult to grasp. Taken together, I propose that the onset of TFC differentiation is defined by three parameters: (i) ecdysone levels, (ii) a threshold of ecdysone sensitivity controlled by IIS, and (iii) a responsive period during which TFC precursors respond to levels of ecdysone.

From these novel and exciting insights on the mechanisms that regulate the onset of TFC differentiation, we can now hypothesize how variation in this trait diverged between *D. moj. sonorensis* and *D. moj. wrigleyi.* Even though changes in the onset of TFC differentiation do not account for differences in ovariole number between the two subspecies, exploring how such variation in the onset of TFC differentiation has evolved may contribute to a better understanding of the developmental processes that regulate ovary development. Differences in the timing of the onset of TFC differentiation between the two subspecies might reflect changes in the timing of the small ecdysone peak, and consequently, in the attainment of critical weight (Figure 5.2A). On the other hand, critical weight could be achieved at the same time, but the threshold amount of ecdysone required to trigger the onset of TFC differentiation may differ between the two subspecies (Figure 5.2B). Ovaries from *D. moj. sonorensis* larvae show an early onset of TFC differentiation and a higher rate of ovary growth when compared with *D. moj. wrigleyi.* Therefore, it is likely that high levels of IIS activity are present in the larval ovary of *D. moj. sonorensis*, which allow TFC precursors to respond to lower levels of ecdysone and induce an early onset of TFC differentiation.

Changes in either the thresholds of hormone sensitivity, the timing of a response period, or the timing of hormone secretion underlie some well-known examples of developmental plasticity (Nijhout, 2003b). Once such case is found amongst the distinct dimorphic worker castes (i.e. soldiers and minor workers) in the ant *Pheidole bicarinata.* In this species, adult ant soldiers produce a pheromone that raises the threshold amount of JH in developing larvae, which reduces their sensitivity to JH and prevents them from developing into soldiers (Wheeler and Nijhout, 1983, 1984). A similar mechanism was shown to regulate the developmental switch between adulthood and dauer larvae (i.e. non-reproductive form that can survive

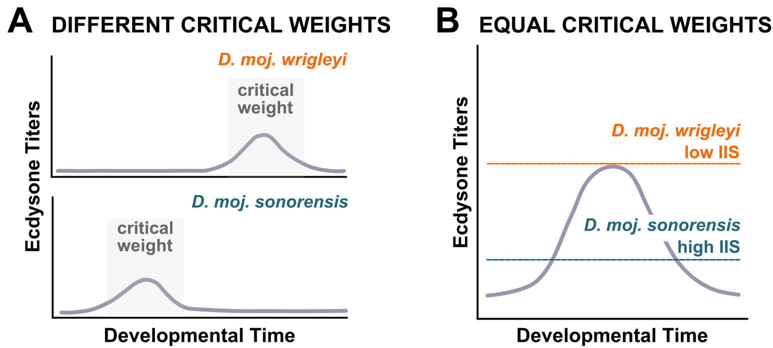

Figure 5.2: Alternative mechanisms might underlie differences in the onset of TFC differentiation between the two *D. mojavensis* subspecies. (A) The small ecdysone peak is secreted at different time points, resulting in differences in the timing of both the attainment of critical weight and the onset of TFC differentiation. (B) Critical weight is attained at the same time. However, ovaries from D. *moj. sonorensis* and *D. moj. wrigleyi* may show substantial differences in IIS activity, and thus, different thresholds of ecdysone sensitivity could exist between the two subspecies. As a result, the onset of TFC differentiation would occur at different time points.

in harsh conditions) in the nematode Caenorhabditis elegans (Schaedel et al., 2012). Here, the authors described the existence of a responsive period during which worms can respond to the hormone dafacronic acid and induce alternative developmental programs. Both crowding and the presence of dauer pheromone raises the threshold of sensitivity to dafacronic acid necessary to induce adult development(Schaedel et al., 2012). Studies in the dung beetle *Onthophagus taurus* have elucidated how thresholds of hormone sensitivity and the responsive period can be altered by social conditions (Emlen and Nijhout, 1999). The *Drosophila* ovary now provides an opportunity to uncover the molecular mechanisms underlying these types of phenomena.

5.3 Plasticity and evolution in ovariole number and body size

Not all traits show the same sensitivity to larval nutrition (Shingleton et al., 2009). One mechanism by which organs can change their nutritional sensitivity is by changing the levels of IIS activity (Tang et al., 2011;

Shingleton and Tang, 2012; Koyama et al., 2013; Shingleton and Frankino, 2013)). For instance, Green and Extavour (2014) found that the ovaries of *D. sechellia* and *D. melanogaster* differ in the levels of phosphorylated Akt, and propose that this variation in the levels of IIS underlies the difference in ovariole number between the two species. Changes in the levels of FOXO activity, a negative regulator of IIS, are also known to mediate the nutritional sensitivity of developing organs (Tang et al., 2011). At low levels of *foxo* mRNA expression, growth of the wing discs is not inhibited even in larvae that are poorly fed, allowing flies to maintain a large wing size when nutrition and IIS is low (Figure 5.3A, B). Whilst moderate levels of *foxo* increase the nutritional sensitivity of the wing, at very high levels of *foxo* expression the wing shows reduced sensitivity to nutrition, and thus, flies bear small wings even when nutrition and IIS are high (Figure 5.3A, B) (Tang et al., 2011). Thus, plotting the degree of nutritional plasticity against IIS activity generates a bell-shaped curve.

Using a similar logic to that used by Tang *et al.* (2011), I hypothesize that the levels of IIS activity regulate the nutritional sensitivity of ovariole number and female body size in the two subspecies of *D. mojavensis.* When larvae were reared across a range of nutritional conditions, female body size in *D. moj. sonorensis* was more sensitive to nutritional variation when compared with *D. moj. wrigleyi* (i.e. slopes of reaction norms were significantly different).Moreover, females of *D. moj. sonorensis* have higher number of ovarioles and larger body size relative to *D. moj. wrigleyi* females. I therefore propose that whole body of *D. moj. sonorensis* females might display moderate levels of IIS signalling, resulting in an increase in its sensitivity to larval nutrition (Figure 5.3C, D). Conversely, females of *D. moj. wrigleyi* might show low levels of IIS activity throughout the body, making them both smaller and less sensitive to nutritional variation (Figure 5.3C, D). In terms of their ovariole number, both subspecies show similar nutritional sensitivity (i.e. slopes of reaction norms were equal). Ovary growth rate in *D. moj. wrigleyi* is significantly reduced in comparison with *D. moj. sonorensis*, and hence, levels of IIS activity in the developing ovary are likely different between the two subspecies. This could occur if the levels of IIS activity were at

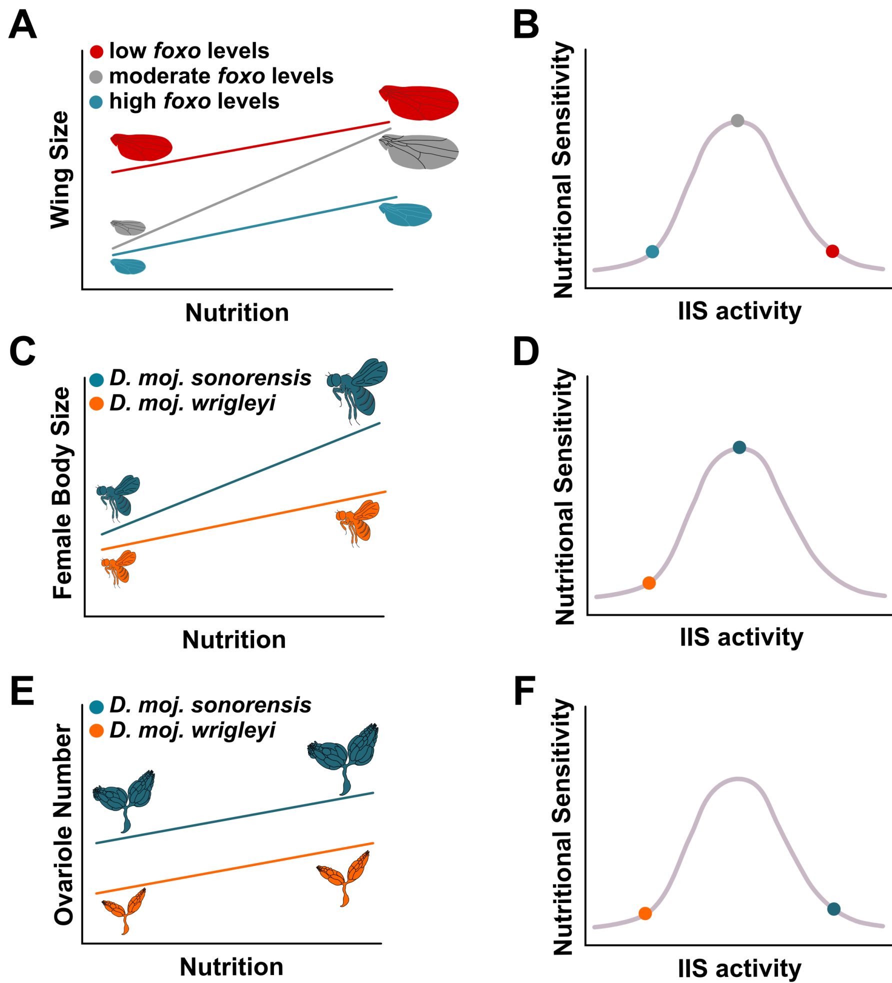

Figure 5.3: The relationship between IIS activity and nutritional sensitivity. (A) Changes in the levels of FOXO activity can account for differences in nutritional sensitivity. Either low or high levels of *foxo* mRNA in the wing discs reduce their sensitivity to nutritional variation. In contrast, wing discs expressing moderate levels of *foxo* mRNA increase their nutritional sensitivity. (B) Therefore, the relationship between nutritional sensitivity and IIS activity shows a bell-shape curve. (C) Different plastic responses and genetic variation for body size between *D. moj. sonorensis* and *D. moj. wrigleyi*. (D) *D. moj. sonorensis* might show moderate levels of IIS activity, resulting in higher nutritional sensitivity for body size. (E) Similar plastic responses, but genetic variation for ovariole number between the two subspecies. (F) Levels of IIS activity may be at opposite extremes of the bell-shape curve, resulting in similar levels of nutritional sensitivity, but different ovary sizes.

opposite extremes of the bell-shaped curve, resulting in similar levels of plasticity but different ovary sizes (Figure 5.3E, F).

My work has focussed on the developmental and physiological mechanisms underlying variation in ovariole number. However, we are beginning to understand the genetic basis of organ and body size. Green and Extavour (2014) further demonstrated that changes in the activity of IIS can account for differences in ovariole number between *D. sechellia* and *D. melanogaster*: high IIS activity in the developing ovary of *D. melanogaster* can promote the formation of a higher number of ovarioles (Green and Extavour, 2014), presumably by increasing the rate of ovary growth. Furthermore, introducing one copy of *D. melanogaster* InR into a *D. sechellia* background increases the number of ovarioles in this species (Green and Extavour, 2014). A similar evolutionary change in IIS activity between *D. moj. sonorensis* and *D. moj. wrigleyi* could underlie differences in ovary growth, which ultimately result in differences in ovariole number.

Together with the current literature on plasticity and evolution of organ size, my work underline that changes in the activity of a major hormonal pathway, the IIS, can account for plastic responses, and potentially facilitate evolutionary diversification among populations and species. Understanding the genetic mechanisms underlying evolutionary changes in the activity of IIS could be a fruitful avenue of research.

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